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(71) Applicant (for all designated States except US): CIBA  
SPECIALTY CHEMICALS HOLDING INC. [CH/CH];  
Klybeckstrasse 141, CH-4057 Basel (CH).

(72) Inventors; and

(75) Inventors/Applicants (for US only): STAMPFER, Wolfgang [AT/AT]; Otto-Loewi Gasse 38, A-8042 Graz (AT).  
KOSJEK, Birgit [AT/AT]; Dorfstrasse 3, A-8750 Judenburg (AT). KROUTIL, Wolfgang [AT/AT]; Dammweg 15, A-8042 Graz (AT). FABER, Kurt [AT/AT]; Kerschhoferweg 25, A-8010 Graz (AT).

(74) Common Representative: CIBA SPECIALTY CHEMICALS HOLDING INC.; Patentabteilung, Klybeckstrasse 141, CH-4057 Basel (CH).

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(54) Title: ALCOHOL DEHYDROGENASES WITH HIGH SOLVENT AND TEMPERATURE STABILITY

(57) Abstract: The invention relates to biocatalysts showing alcohol dehydrogenase activity obtainable from *Rhodococcus ruber*, their preparation, their use in the oxidation of secondary alcohols and/or the reduction of ketones, as well as nucleic acids coding for these alcohol dehydrogenases and microorganisms transformed with nucleic acids coding for these biocatalysts and their use for producing the biocatalyst or oxidizing secondary alcohols and/or reducing ketones.

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## Alcohol dehydrogenases with high solvent and temperature stability

### Summary of the invention

The invention relates to biocatalysts showing alcohol dehydrogenase activity, their preparation, their use in the oxidation of secondary alcohols and/or the reduction of ketones, as well as nucleic acids coding for these alcohol dehydrogenases and microorganisms transformed with nucleic acids coding for these biocatalysts and their use for producing the biocatalyst.

### Background of the invention

The Oppenauer oxidation (Op-Ox) and the inverse reduction, the Meerwein-Ponndorf-Verley reduction (MPV-Red), are well-established oxidation or reduction processes for alcohols or ketones, respectively. The reactions are possible without requiring toxic heavy metals as catalysts (see, for example, J. March, *Advanced Organic Chemistry*, 3<sup>rd</sup> Ed., John Wiley 1985, pp 1058, 813; ISBN 04718547-7; C. F. deGraauw, J. A. Peters, H. van Bekkum, J. Huskens, *Synthesis*, 1994, 1007-17; and S. D. Burke, D. L. Danheiser, *Handbook of Reagents for Organic Synthesis, Oxidizing And Reducing Reagents*, Wiley 1994; ISBN 0471979260).

A disadvantage is that the strongly basic reaction conditions lead to undesired aldol-type side reactions (see K. G. Akamanchi, B. A. Chaudhari, *Tetrahedron Lett.* **38**, 6925-8 (1997)). Substrates sensitive to basic conditions cannot be reacted without decomposition (see T. Ooi, Y. Itagaki, T. Miura, K. Maruoka, *Tetrahedron. Lett.* **40**, 2137-8 (1999)). Asymmetric variants of the MPV-Red that employ chiral transition metal catalysts for enantioselective hydride transfer have been tested only on few model substrates and resulted in preparatively inacceptably low stereoselectivities (see F. Touchard, M. Bernard, F. Fache, F. Debbecq, V. Guiral, P. Sautet, M. Lemaire, *J. Org. Met. Chem.* **567**, 133-6 (1998); und E. Breyse, C. Pinel, M. Lemaire, *Tetrahedron: Asymmetry* **9**, 897-900 (1998)).

In contrast, biocatalytic methods have the advantage that they can be led under mild conditions, e.g. at room temperature and approximately neutral pH in aqueous media (see K. Faber, *Biotransformations in Organic Chemistry* 4<sup>th</sup> Ed., Springer Verlag, , Heidelberg 2000; ISBN 3-540-61688-8). An additional valuable property of biocatalysts is their normally high intrinsic stereoselectivity. In addition, the desired reaction usually takes place without side

reactions. Biocatalytic redox processes on the basis of isolated alcohol dehydrogenases, however, require the presence of expensive cofactors, such as  $\text{NAD}^+/\text{NADH}$  or  $\text{NADP}^+/\text{NADPH}$ . The recycling of these substrates is difficult and expensive (see W. Hummel, Adv. Biochem. Eng./Biotechnol. **1997**, 58, 145-184). One improvement of such processes is based on the presence of a second enzyme which, in the presence of a reducible or oxidizable component or cofactor, respectively, allows to recover the co-factor ("enzyme-coupled system", see W. Hummel, B. Riebel, Ann. N. Y. Acad. Sci. **1996**, 799, 713-6). This variant, however, renders the process relatively complex and difficult to handle, as it is limited to such additives which are accepted as auxiliary component by the second enzyme. In addition, the concentrations of the substrates and the enzymes must be harmonized precisely for resulting in preparatively acceptable reaction rates. Furthermore, isolated enzymes usually have relatively short half lives under operating conditions. In order to achieve complete reaction into one direction, in the case of oxidation a carbonyl compound is added as reducible co-substrate in large molar excess, in the case of reduction a secondary alcohol as oxidizable co-substrate is added in large molar excess. This often results in difficulties especially with enzyme stability, as well as enzyme inhibition by the co-substrate.

If whole cells are used as biocatalysts in the stadium of fermentation, a lower addition of co-factors is required. In addition the cells are capable of recycling the co-factors themselves. However, the cells react very sensitively on high concentrations of organic substrates (substrate inhibition, solvent deactivation). For this reason, the biochemical MPV-Red and Op-Ox are limited to fermentative cells systems and low (co-)substrate concentrations (see G. Fantin, M. Fogagnolo, A. Medici, P. Pedrini, S. Fontana, Tetrahedron: Asymmetry **2000**, 11, 2367-73). In general, the substrate concentrations are below 0.15 mol/l and the co-substrate concentrations below 3 % (v/v) (see K. Nakamura, Y. Inoue, T. Matsuda, I. Misawa, J. Chem. Soc. Perkin Trans. I **1999**, 2397-2402; and A. Goswami, R. L. Bezbaruah, J. Goswami, N. Borthakur, D. Dey, A. K. Hzarika, Tetrahedron: Asymmetry **2000**, 11, 3701-9).

Reductions of ketones to the respective alcohols have also been conducted with acetone-pulverized *Geotrichum candidum* cells (see K. Nakamura, T. Matsuda, J. Org. Chem. **1998**, 63, 8957-64). However, in this case, the cellular redox systems was partially inactivated during the freeze-drying of the cells. This required the addition of expensive redox-cofactors, such as  $\text{NAD}^+/\text{NADH}$  or  $\text{NADP}^+/\text{NADPH}$ , for subsequent use as biocatalyst. The addition of

(reducing) isopropyl alcohol as co-substrate was limited to about 3 % (v/v) in that case. Due to the relatively low concentration of this organic co-solvent, the concentration of lipophilic ketone substrates could only be adjusted to a maximum of 0.4 mol/l.

In view of all these unsatisfying results which constitute an unmet need, there remains the problem to find novel, more efficient catalytic systems offering more ease of use, which diminish or remove the mentioned and other unfavorable disadvantages and allow to conduct redox reactions of alcohols to oxo (especially keto) compounds or the inverse reaction in an especially advantageous way.

It is thus an object of the present invention to provide novel catalytic systems that allow for avoiding the mentioned disadvantages and that have advantageous properties that allow for improved biocatalytic reduction of ketones and/or oxidation of secondary alcohols, respectively.

#### General Description of the invention

The invention relates to a biocatalyst, especially an enzyme, preferably in (at least partially) purified form, which biocatalyst has alcohol dehydrogenase activity and which can be obtained from *Rhodococcus ruber*.

It has been found that this enzyme has unexpected and unique properties in comparison to other, known enzymes having the same type of activity. Especially, the novel enzyme has high temperature stability and in addition is capable of maintaining its activity in the presence of high concentrations of organic solvents (such as aromatic or aliphatic hydrocarbons, e.g. toluene, hexane) other than the co-substrates in up to 95, preferably up to 98 % concentration (v/v). This especially allows for leading the oxidation or reduction reactions under conditions of high temperature and especially in the presence of high co-substrate concentrations (in the case of oxidation of alcohols, the presence of high ketone concentrations; in the case of reduction of ketones, the presence of high alcohol concentrations). It therefore allows for production on an industrially useful scale, especially avoiding many of the disadvantages mentioned for the reactions formerly described. Among the further advantages, a prolonged storage stability can be mentioned, as well as a high operational stability of catalytic activity. The enzyme is also capable of working also in the

presence of complex-forming agents that usually would cause removal of bound catalytic metals like  $Zn^{2+}$ .

#### Description of the Figures

**Fig. 1:** Native gel analysis at different steps of the purification protocol of *sec*-alcohol dehydrogenase A. Lanes: Lane 1 – crude cell extract; lane 2: batch DEAE cellulose; lane 3: Phenyl Sepharose; lane 4: UNO Q6; lane 5: Blue Sepharose; lane 6: Superdex 200.

**Fig. 2:** SDS-PAGE analysis at different steps of the purification protocol of *sec*-alcohol dehydrogenase A. Lanes: Lane 1 – crude cell extract; lane 2: batch DEAE cellulose; lane 3: Phenyl Sepharose; lane 4: UNO Q6; lane 5: Blue Sepharose; lane 6: Superdex 200; lane 7: low molecular weight standard.

#### Detailed description of the invention

The following are preferred embodiments of the invention:

The invention especially relates to an biocatalyst with alcohol dehydrogenase activity, especially an enzyme, preferably in (at least partially) purified form, which has alcohol dehydrogenase activity, especially stereospecific alcohol dehydrogenase activity in the oxidation of secondary alcohols or the reduction of ketones; and which can be obtained from a naturally occurring microorganism, especially *Rhodococcus*, especially *Rhodococcus ruber* DSM 44541 –called *Rhodococcus ruber* DSM 14855 hereafter, in accordance with the number of the Budapest Treaty deposit (see below).

The biocatalyst (or “enzyme”) according to the invention is preferably present or used in purified form.

The invention also relates to a corresponding biocatalyst with alcohol dehydrogenase activity, obtained by recombinant technology (recombinant biocatalyst).

A further embodiment of the invention relates to the use of a biocatalyst with alcohol dehydrogenase activity according to the invention in the oxidation of secondary alcohols and/or the reduction of ketones (process of or according to the invention, hereinafter).

The process of the invention can be used especially for separating mixtures of stereoisomers with respect to a center of chirality by kinetic resolution, if one stereo-isomer of an alcohol is specifically oxidized, or for the stereo-specific production of secondary alcohols having a specific chiral form from ketones.

Still a further embodiment relates to nucleic acids coding for such a biocatalyst with alcohol dehydrogenase activity, especially recombinant nucleic acids.

Another embodiment relates to microorganisms transformed with a nucleic acid coding for such a biocatalyst with alcohol dehydrogenase activity.

Further, the invention relates to the use of the mentioned microorganisms, especially host cells, in the production of said biocatalyst with alcohol dehydrogenase activity, as well as their use in the catalysis of the oxidation of secondary alcohols or the reduction of ketones, especially as shown in reaction scheme (A) below.

Unless indicated otherwise, the general terms, symbols and names used in the description of the present invention preferably have the following meanings (where more specific definitions, in each case separately, or in combination, may be used to replace more general terms in order to define more preferred embodiments of the invention):

The invention especially relates to a biocatalyst with alcohol dehydrogenase activity, especially an enzyme, preferably in purified form, which has alcohol dehydrogenase activity and which can be obtained from *Rhodococcus ruber*, especially *Rhodococcus ruber* DSM 14855. That the biocatalyst has alcohol dehydrogenase activity, is not intended to mean that other activities (be it of enzymatic, regulatory or any other kind) are excluded within the present disclosure.

The terms "biocatalyst of the invention" or "enzyme of the invention", where used herein, relates to a biocatalyst having alcohol dehydrogenase activity, especially an enzyme with said activity, most especially alcohol dehydrogenase "ADH-A", as described below. If not stated otherwise, all these terms include not only the naturally occurring, "authentic" sequence of a polypeptide of the invention, which are the preferred embodiments of the



invention, but also all mutants, variants and fragments thereof which exhibit the alcohol dehydrogenase activity, preferably with the same stereoselective activity as the natural enzyme.

An enzyme of the invention preferably has one, more preferably two, most preferably three or more of the following properties:

- (i) Molecular weight on denaturing SDS polyacrylamide gel after electrophoresis: 30 to 45 kDa, especially about 38 kDa.
- (ii) Molecular weight according to size exclusion chromatography (especially under the conditions mentioned in the examples) on Superdex 200: 55 to 69 kDa, especially about 62 kDa.
- (iii) pH optimum in the reduction of ketones, especially of acetophenone in the presence of NADH: pH 6 to pH 7.
- (iv) pH optimum in the oxidation of alcohols, especially of 1-phenylethanol in the presence of NAD<sup>+</sup>: pH 8.5 to pH 9.5.
- (v) Temperature optimum in the reduction of ketones, especially acetophenone in the presence of NADH: between 43 and 65 °C.
- (vi) Temperature optimum in the oxidation of secondary alcohols, especially 1-phenylethanol in the presence of NAD<sup>+</sup>: between 43 and 65 °C.
- (vii) Temperature stability under the conditions just given for the temperature optimum, specifically with the substrates and co-substrates mentioned after "especially": at 50 °C less than 35 % activity loss during 24 hours.
- (viii) No activity change in the presence of EDTA (especially up to 5 mM concentration of EDTA) in an activity test at pH 7.5, 20 min incubation.
- (ix) Presence of Zn<sup>2+</sup> bound to the molecule.
- (x) Stability, especially alcohol dehydrogenase activity also in the presence of up to 50, preferably up to 80 percent by volume of isopropanol.
- (xi) Stability, especially alcohol dehydrogenase activity also in the presence of up to 20, preferably up to 50 percent by volume of acetone.
- (xii) Presence of the partial sequence EVGADAAAR (SEQ ID No: 1) within the total sequence of at least one polypeptide forming the whole or part of the enzyme.
- (xiii) Presence of the partial sequence TD[L/I]FEVVA[L/I]AR (SEQ ID NO: 2) within the total sequence of at least one polypeptide forming the whole or part of the enzyme, where [L/I] is leucine or isoleucine.

- (xiv) Presence of the partial sequence SGAGAADA[L/I]R (SEQ ID NO: 3) within the total sequence of at least one polypeptide forming the whole or part of the enzyme, where [L/I] is leucine or isoleucine.
- (xv) Presence of the partial sequence V[L/I]AVD[L/I]DDDE (SEQ ID NO: 4) within the total sequence of at least one polypeptide forming the whole or part of the enzyme, where [L/I] is leucine or isoleucine.
- (xvi) Presence of the partial sequence V[L/I]AVD[L/I]DDDXRX (SEQ ID NO: 5) within the total sequence of at least one polypeptide forming the whole or part of the enzyme, where [L/I] is leucine or isoleucine and X stands for an unidentified amino acid.
- (xvii) Presence of the partial sequence [TD/DT] [L/I]MEVVA[L/I]AR (SEQ ID NO: 6 (either with TD in the beginning = at the amino terminus or with DT in the beginning) within the total sequence of at least one polypeptide forming the whole or part of the enzyme where the sequence in brackets is selected from the two alternatives mentioned therein and [L/I] is isoleucine or leucine.

Especially preferred among these properties are those mentioned under (x) and/or (xi).

The term "within the total sequence of at least one polypeptide forming the whole or part of the enzyme" refers to the possibility that the biocatalyst/enzyme according to the invention may consist of one subunit (then it is formed from one polypeptide) or more than one (identical or different) subunits (then it is formed from the corresponding number of polypeptides).

More preferred is an enzyme of the invention wherein one of the partial sequences mentioned under (xii) to (xvii) is present or wherein one of the amino acids mentioned in any of these sequences is exchanged against a different amino acid, preferably by a conservative replacement, e.g. of lipophilic against lipophilic, basic against basic, acidic against acidic, polar against polar amino acids, or the like.

Still more preferred is an enzyme the peptide sequence or, if more than one different subunits are present, sequences of which comprise a partial sequence selected from two, more preferably 3, even more preferably 4, still most preferably 5, most preferably 6 of the sequences mentioned above under (xii) to (xvii).

The enzyme of the invention is isolated (purified) from the microorganism by methods that are, *per se*, well known in the art, in particular by the methods described in the examples or methods analogous thereto, the whole purification method also forming an embodiment of the invention.

The Alcohol dehydrogenase activity is preferably determined by oxidation of 1-phenylethanol (6.6  $\mu$ M) and addition of 10 mM NAD<sup>+</sup> (testing conditions: 30 °C, 10  $\mu$ M Tris-buffer, pH 7.5, 10 min, conversion by GC-analysis), or as described in the Examples.

A biocatalyst of the invention is obtainable from a naturally occurring microorganism fermentable by a process comprising inoculating a selection medium with natural samples such as soil, water, or plant silage, preferably a hydrocarbon, e.g. hexane. Apart from the carbon source, the selection medium also contains all essential ingredients necessary for allowing growth of microorganisms, such as mineral salts, N-sources, and trace elements.

A naturally occurring microorganism having alcohol dehydrogenase activity can be obtained by a process known in the art, for example by isolation from a natural source, such as Rhine water.

In order to obtain the purified enzyme of the invention, the microorganism having alcohol dehydrogenase activity, especially *Rhodococcus ruber* DSM 14855, is cultivated in an aqueous nutrient medium, e.g. comprising yeast extract, peptone, glucose and mineral salts (e.g. 10 g/l yeast extract 10 g/l peptone, 2 g/l NaCl, 0.15 g/l MgSO<sub>4</sub> • 7H<sub>2</sub>O, 1.3 g/l NaH<sub>2</sub>PO<sub>4</sub>, 4.4 g/l K<sub>2</sub>HPO<sub>4</sub>) for some, e.g. three, days. Cell growth is followed by determination of the optical density *via* absorption, e.g. at 546 nm.

The cells are disrupted, the biomass is removed and the cell-free extract is obtained. The fermentation time is so selected that optimum titers with respect to alcohol dehydrogenase activity are achieved.

When the cell density has reached an adequate value, the cultivation is discontinued. The culture broth is separated off in known manner, e.g. by centrifugation, and the sedimented cells are broken down in customary manner, e.g. by shaking with fine glass beads, by ultrasound treatment, or using a French press. Insoluble cell components and, if used, glass

beads, are removed, e.g., by centrifugation, and the residue is used as the enzyme source (crude extract). The residue, as an alcohol dehydrogenase activity-containing crude extract, can be used directly in the process according to the invention. Advantageously, however, in order to remove nucleic acids (viscous solutions) and other impurities or interfering components (e.g. other dehydrogenases with lower steric selectivity) the crude extract is subjected to further purification in order to obtain the enzyme of the invention in purified form. Preferably, the crude cell extract is subjected to one or more purification steps that, as such, are known in the art in order to remove interfering components from the extract.

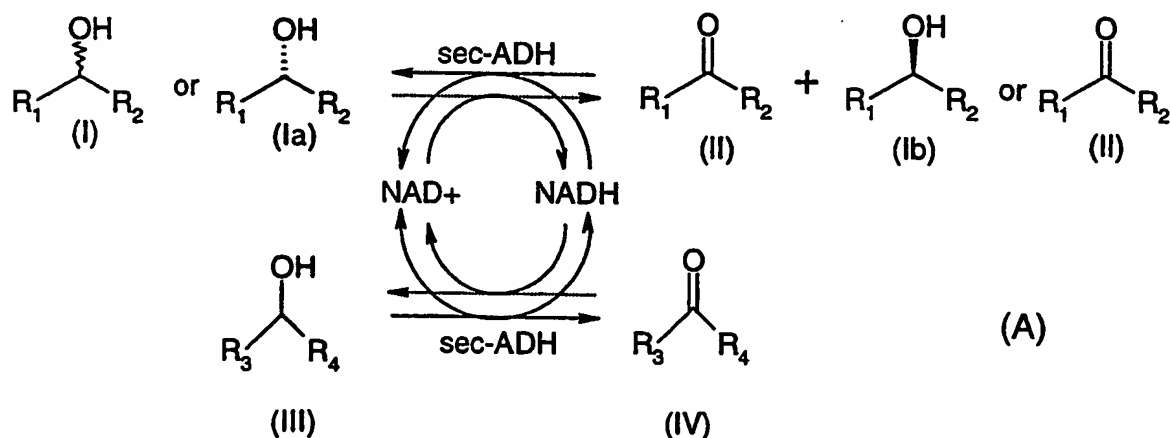
A preferred method makes use of batch pretreatment with a cation exchanger, e.g. DEAE cellulose, and subsequent chromatographic (especially FPLC) separation first by Hydrophobic Interaction Chromatography, e.g. over Phenyl Sepharose, advantageously after removing precipitations formed in the presence of  $(\text{NH}_4)_2\text{SO}_4$ . With increasing  $(\text{NH}_4)_2\text{SO}_4$  content of the eluent, enzyme activity is eluted. The active fractions are further separated by anion exchange chromatography, preferably on an UNO Q column (BioRad). Elution by eluents with increasing sodium chloride content yields again active fractions with alcohol dehydrogenase activity. The interesting fractions are then further purified on a separation matrix comprising binding places for adenylyl-comprising cofactors, e.g. carrying the dye Cibacron Blue F3G-A or other moieties allowing for such affinity chromatography, especially using Blue Sepharose Cl-6B, this innovative material allowing a surprisingly good further purification, although surprisingly no binding takes place. This adds to the information showing surprising properties of the enzyme according to the invention. The next step is size exclusion chromatography, e.g. using a Superdex 200 column. Here the enzyme of the invention elutes in fractions corresponding to a molecular weight between 55 to 69 kDa, with an average of about 62 kDa.

The term "purified" means preferably "in at least partially purified form" or "in enriched form" or, more preferably, purified in the stricter sense, that is, in practically isolated form (especially with more than 50, most especially more than 95 % purity by weight compared to other peptides present).

A corresponding biocatalyst with alcohol dehydrogenase activity, obtained by recombinant technology, then called a recombinant biocatalyst of the invention, (or also a biocatalyst from natural sources other than *Rhodococcus ruber* DSM 14855), preferably is defined as follows.

The sequence of said biocatalyst may comprise deletions, insertions, terminal additions or exchanges (especially conservative exchanges, e.g. of lipophilic against lipophilic, basic against basic, acidic against acidic, polar against polar amino acids, or the like) of amino acids (preferably of up to 20, in case of terminal additions up to 1000; more preferably of up to 5, in case of terminal additions of up to 200 amino acids, respectively), or any combination of such changes, when compared to the sequence of the enzyme as purified in the examples or possible different subunits thereof, as long as the basic activity (alcohol dehydrogenase activity, especially with the substrates (preferably 1-phenylethanol or acetophenone) and co-substrates mentioned in the examples) is still present, especially in connection with one or more of the additional advantageous properties mentioned for a biocatalyst according to the invention. Also, modified amino acids (with different structures than the 20 amino acids directly derivable from the genetic code) which may be modified during translation or post-translationally, may be present, e.g. 1 to 20, more preferably 1 to 5 such amino acids.

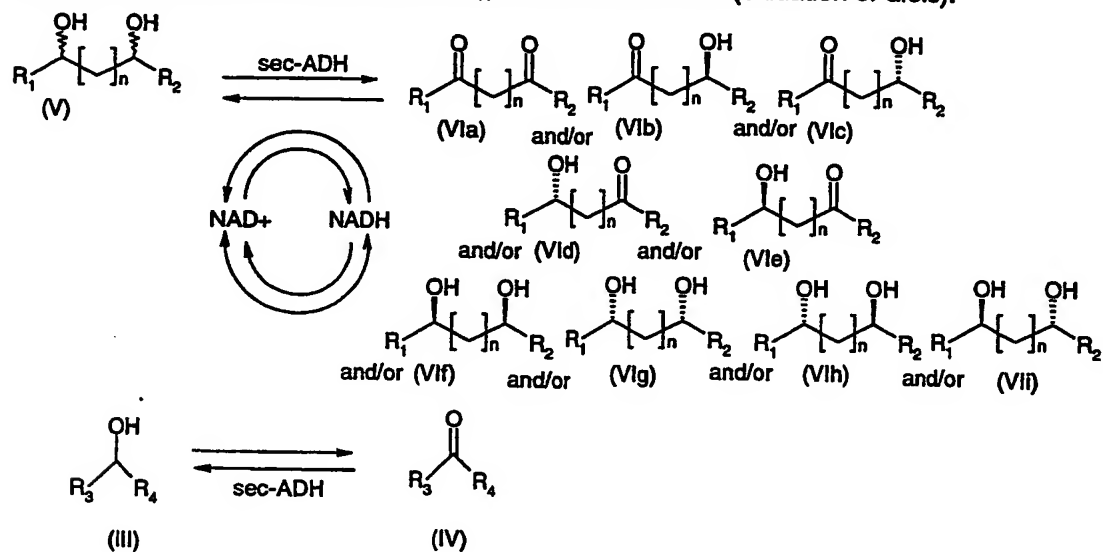
In a preferred embodiment of the use a biocatalyst with alcohol dehydrogenase activity in the oxidation of secondary alcohols or the reduction of ketones is the use in catalyzing the following reactions (reaction scheme (A)):



wherein, in formula I, Ia and Ib, R<sub>1</sub> and R<sub>2</sub> are two different moieties from the group consisting of unsubstituted or substituted alkyl, unsubstituted or substituted alkenyl, unsubstituted or substituted alkynyl, unsubstituted or substituted cycloalkyl, unsubstituted or substituted aryl and unsubstituted or substituted heterocyclyl, or R<sub>1</sub> and R<sub>2</sub> together form an unsubstituted or substituted bridge; and

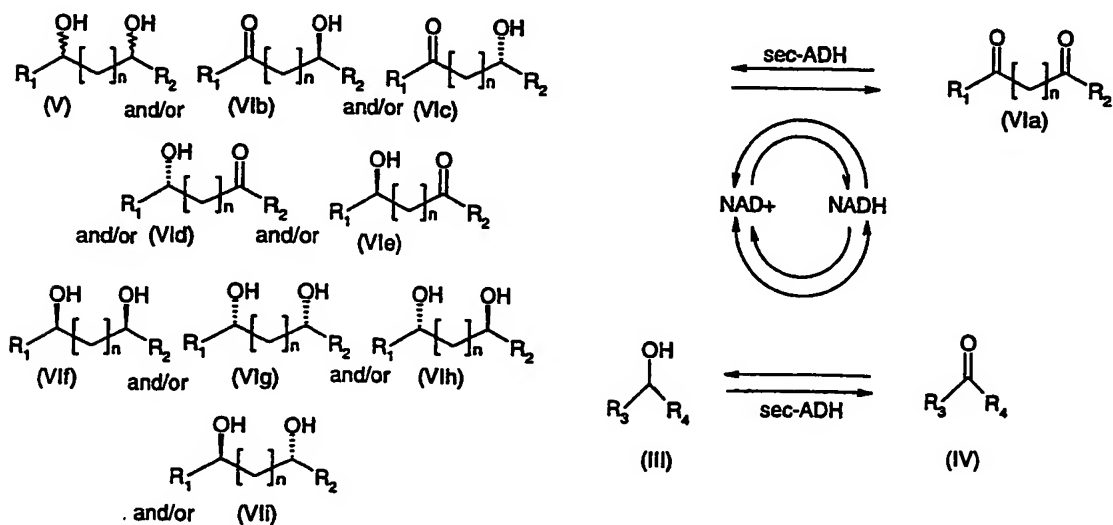
in formula III and formula IV,  $R_3$  and  $R_4$  are two different or preferably two identical lower alkyl or aryl moieties, or together form a bridge.

A further preferred embodiment of the present invention comprises the use of a biocatalyst with alcohol dehydrogenase activity in the oxidation of secondary alcohols, preferably of secondary diols, or reduction of ketones, preferably of diketones, as shown in following reactions (reaction schemes (B) and (C)): reaction scheme B (oxidation of diols):



and

reaction scheme C (reduction of diketones):



wherein

n is a whole number in the range from 0 to 20, preferably from 2 to 20, and more preferably from 2 to 6, and R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and R<sub>4</sub> have the same meaning as given above for compounds in reaction scheme A.

The term "lower" defines a moiety with up to and including maximally 7, especially up to and including maximally 4, carbon atoms, said moiety being branched or straight-chained. Lower alkyl, for example, is ethyl, n-propyl, sec-propyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, n-hexyl or n-heptyl or most preferably methyl.

"Substituted", wherever used for a moiety, means that one or more hydrogen atoms in the respective molecule, especially up to 5, more especially up to three, of the hydrogen atoms are replaced by the corresponding number of substituents which preferably are independently selected from the group consisting of alkyl, especially lower alkyl, for example methyl, ethyl or propyl, fluoro-lower alkyl, for example trifluoromethyl, C<sub>6</sub>-C<sub>16</sub>-aryl, especially phenyl or naphthyl (where C<sub>6</sub>-C<sub>16</sub>-aryl, especially phenyl or naphthyl, is unsubstituted or substituted by one or more, especially up to three moieties selected from halogen, carboxy, lower alkoxy, carbonyl, hydroxy, lower alkoxy, phenyl-lower alkoxy, lower alkanoyloxy, lower alkanoyl, amino, N-lower alkylamino, N,N-di-lower alkylamino, N-phenyl-lower alkylamino, N,N-bis(phenyl-lower alkyl)-amino, lower alkanoylamino, fluoro-lower alkyl, e.g. trifluoromethyl, and sulfo), C<sub>3</sub>-C<sub>10</sub>-cycloalkyl (that is unsubstituted or substituted by one or more, especially up to three moieties selected from halogen, carboxy, lower alkoxy, carbonyl, hydroxy, lower alkoxy, phenyl-lower alkoxy, lower alkanoyloxy, lower alkanoyl, amino, N-lower alkylamino, N,N-di-lower alkylamino, N-phenyl-lower alkylamino, N,N-bis(phenyl-lower alkyl)-amino, lower alkanoylamino, fluoro-lower alkyl, e.g. trifluoromethyl, and sulfo), heterocyclyl that is unsaturated, saturated or partially saturated, is mono-, bi- or tri-cyclic and has 4 to 16 ring atoms, where instead of one or more, especially one to four, carbon ring atoms the corresponding number of heteroatoms are present (within the chemically possible limits) selected from nitrogen, oxygen and sulfur (with said heterocyclyl unsubstituted or substituted by one or more, especially up to three moieties selected from halogen, carboxy, lower alkoxy, carbonyl, hydroxy, lower alkoxy, phenyl-lower alkoxy, lower alkanoyloxy, lower alkanoyl, amino, N-lower alkylamino, N,N-di-lower alkylamino, N-phenyl-lower alkylamino, N,N-bis(phenyl-lower alkyl)-amino, lower alkanoylamino, fluoro-lower alkyl, e.g. trifluoromethyl, and sulfo), hydroxy, lower alkoxy, for example methoxy, phenyl-lower alkoxy, lower alkanoyloxy,

amino, N-lower alkylamino, N,N-di-lower alkylamino, N-phenyl-lower alkylamino, N,N-bis-(phenyl-lower alkyl)-amino, lower alkanoylamino, carbamoyl-lower alkoxy, N-lower alkyl-carbamoyl-lower alkoxy or N,N-di-lower alkylcarbamoyl-lower alkoxy, amino, mono- or di-lower alkylamino, lower alkanoylamino, carboxy, lower alkoxycarbonyl, phenyl-, naphthyl- or fluorenyl-lower alkoxycarbonyl, such as benzyloxycarbonyl, lower alkanoyl, sulfo, lower alkanesulfonyl, for example methanesulfonyl ( $\text{CH}_3\text{-S(O)}_2\text{-}$ ), phosphono ( $\text{-P(=O)(OH)}_2\text{-}$ ), hydroxy-lower alkoxy phosphoryl or di-lower alkoxyphosphoryl, carbamoyl, mono- or di-lower alkylcarbamoyl, sulfamoyl and mono- or di-lower alkylaminosulfonyl. It goes without saying that substituents are only at positions where they are chemically possible, the person skilled in the art being able to decide (either experimentally or theoretically) without inappropriate effort which substitutions are possible and which are not. Where more than one substituent is present, the substituents, if not indicated otherwise, are selected independently from each other.

Alkyl preferably has up to 24, more preferably up to 12 carbon atoms and, if possible in view of the number of carbon atoms, is linear or branched one or more times; preferred is lower alkyl, especially  $\text{C}_1\text{-C}_4\text{-alkyl}$ . Alkyl can be substituted or unsubstituted, especially by one or more, more especially up to 3, of the substituents mentioned above under "substituted". Unsubstituted alkyl, especially lower alkyl, is one preferred embodiment.

Alkenyl is preferably a moiety with one or more double bonds and preferably has 2 to 20, more preferably up to 12, carbon atoms; it is linear or branched one or more times (as far as possible in view of the number of carbon atoms). Preferred is  $\text{C}_2\text{-C}_7\text{-alkenyl}$ , especially  $\text{C}_3\text{-C}_4\text{-alkenyl}$ , such as allyl or crotyl. Alkenyl can be unsubstituted or substituted, especially by one or more, more especially up to three, of the substituents mentioned above under „substituted“. Substituents such as amino or hydroxy (with free dissociable hydrogen) preferably are not bound to carbon atoms that participate at a double bond, and also other substituents that are not sufficiently stable are preferably excluded. Unsubstituted alkenyl, in particular  $\text{C}_2\text{-C}_7\text{-alkenyl}$ , is especially preferred.

Alkynyl is preferably a moiety with one or more triple bonds and preferably has 2 to 20, more preferably up to 12, carbon atoms; it is linear or branched one or more times (as far as possible in view of the number of carbon atoms). Preferred is  $\text{C}_2\text{-C}_7\text{-alkynyl}$ , especially  $\text{C}_3\text{-C}_4\text{-alkynyl}$ , such as ethynyl or propin-2-yl. Alkynyl can be unsubstituted or substituted, especially



by one or more, more especially up to three, of the substituents mentioned above under „substituted“. Substituents such as amino or hydroxy (with free dissociable hydrogen) preferably are not bound to carbon atoms that participate at a triple bond, and also other substituents that are not sufficiently stable are preferably excluded. Unsubstituted alkynyl, in particular C<sub>2</sub>-C<sub>7</sub>-alkynyl, is especially preferred.

Aryl preferably has a ring system with not more than 20 carbon atoms, especially not more than 14 carbon atoms; is preferably mono-, bi- or tricyclic; and is unsubstituted or substituted by one or more, especially up to three, substituents, preferably as defined above under “substituted”. For example, aryl is selected from the group consisting of phenyl, naphthyl, indenyl, azulenyl and anthryl, each of which is unsubstituted or substituted; preferably from phenyl or 1- or 2-naphthyl, each unsubstituted or substituted by one or more, preferably up to 5, substituents as defined above under “substituted”. In a further embodiment of the present invention aryl is for example a metal complex, such as ferrocen.

Heterocyclyl is preferably a heterocyclic radical that is unsaturated, saturated or partially saturated, and is preferably a monocyclic or, in a broader aspect of the invention, bi- or tricyclic moiety; it has preferably 3 to 24, especially 4 to 16 carbon atoms, where instead of one or more, especially one to four, carbon ring atoms the corresponding number of heteroatoms are present, especially (within the chemically possible limits) selected from nitrogen, oxygen and sulfur; and where heterocyclyl is unsubstituted or substituted by one or more, especially up to three, substituents as defined above under “substituents”. Preferably heterocyclyl is selected from the group consisting of oxiranyl, aziranyl, 1,2-oxathiolanyl, imidazolyl, thienyl, furyl, tetrahydrofuryl, pyranal, thiopyranal, thianthrenyl, isobenzofuranyl, benzofuranyl, chromenyl, 2*H*-pyrrolyl, pyrrolyl, pyrrolinyl, pyrrolidinyl, imidazolyl, imidazolidinyl, benzimidazolyl, pyrazolyl, pyrazinyl, pyrazolidinyl, pyranol, thiazolyl, isothiazolyl, dithiazolyl, oxazolyl, isoxazolyl, pyridyl, pyrazinyl, pyrimidinyl, piperidyl, piperazinyl, pyridazinyl, morpholinyl, thiomorpholinyl, indoliziny, isoindolyl, 3*H*-indolyl, indolyl, benzimidazolyl, cumaryl, indazolyl, triazolyl, tetrazolyl, purinyl, 4*H*-chinoliziny, isochinolyl, chinolyl, tetrahydrochinolyl, tetrahydroisochinolyl, decahydrochinolyl, octahydroisochinolyl, benzofuranyl, dibenzofuranyl, benzothiophenyl, dibenzothiophenyl, phthalazinyl, naphthyridinyl, quinoxalyl, quinoxalinyl, quinoxalinyl, cinnolyl, pteridinyl, carbazolyl,  $\beta$ -carbolinyl, phenanthridinyl, acridinyl, perimidinyl, phenanthrolinyl, furazanyl, phenazinyl, phenothiazinyl, phenoxazinyl, chromenyl, isochromanyl and chromanyl, where each of these moieties is

unsubstituted or substituted as described above, especially by one or more moieties selected from lower alkyl, especially methyl, lower alkoxy, especially methoxy, halogen, especially fluoro, chloro, bromo or iodo, and halogen-lower alkyl, especially trifluoromethyl.

Cycloalkyl preferably has 3 to 12, more preferably 3 to 8 carbon atoms and is, for example, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl or cycloheptyl; it is unsubstituted or substituted with one or more, especially up to 3, substituents, preferably as defined above under "substituted".

An unsubstituted or substituted bridge formed from  $R_1$  and  $R_2$  in formula I, Ia, Ib or II is preferably a bridge formed by 2 to 12 carbon atoms that, together with the binding atom in formula I, Ia, Ib or II forms a ring; where the bridge may contain one or more double and/or triple bonds at places other than the binding carbon atom (then the bridge has at least three carbon atoms) or is preferably saturated. The substituents are preferably chosen from 1 or more, especially up to three, substituents as defined under "substituents". Most preferred is a  $C_2$ - $C_7$ -alkylen chain, such as ethylene, propylene, n-butylene, n-hexylene or n-heptylene, each of which is substituted by one or more, especially 1 or 2, of the moieties defined above under "substituted", or preferably is unsubstituted.

As far as compounds (including alcohols, ketones, substrates, co-substrates and the like) are mentioned herein, this includes also the corresponding salts or solvate salts thereof, if salt-forming compounds are present, as well as the corresponding solvates. Saltforming groups are especially basic groups, such as amino groups, or acidic groups, such as carboxy groups. In the case of acidic groups, the corresponding metal salts, such as alkaline metal salts, e.g. sodium or potassium salts, or alkaline-earth metal salts, such as calcium salts, or salts with nitrogen bases, such as ammonium-, tri-lower alkylammonium, pyridinium salts or the like, can be present; in the case of basic groups, the corresponding acid addition salts may be present, e.g. with inorganic acids, such as sulphuric acid or hydrogen halides, such as HCl or HBr, or with organic acids, e.g. carboxylic acids, such as acetic acid, or sulfonic acids, e.g. methane sulfonic acid.

In the context of the present invention, secondary alcohols have at least a single hydroxy group. Examples for preferred secondary alcohols are at least a single hydroxy group carrying compounds. The secondary alcohols of the present invention are for example

isoprenoids, such as mono-, di- or tri-terpenes, e.g. geraniol, isoborneol, ipsenol, menthol (especially ( $\pm$ )-menthol), nerolidol, hemanulcin, taxol or lanosterol, or steroids, such as cholestan-3-ol, cholesterol, ergosterin, stigmasterin, cholic acids, vitamin D<sub>2</sub>, vitamin D<sub>3</sub>, androsterone, testosterone, estrone, 17 $\beta$ -estradiol, estriol, cortisol, corticosterone, aldosterone, triamincolone, digitoxigenin, strophanthidine, ouabagenine, scillaridine or bufotalin; di-lower alkyl- or lower alkyl-lower alkenyl methanols, such as isopropanol, butane-2-ol, n-hexane-2-ol, n-heptane-2-ol, n-octane-2-ol, n-nonane-2-ol, 3-octanol, 5-methyl-2-heptanol, 3-octen-2-ol or 6-methyl-hex-5-en-2-ol; or n-decane-2-ol; R-, S- or R,S-1-phenyl-1-ethanol, benzhydrol, R-, S- or R,S-1-(1- or 2-naphthyl)-1-ethanol, R-, S- or R,S-1-phenyl-2-butanol, wherein the naphthyl or phenyl moieties are unsubstituted or substituted by one or more, preferably up to 5, substituents independently selected from lower alkoxy, such as methoxy, lower alkyl, such as methyl, nitro, cyano, halogen, such as fluorine, chlorine, bromine or iodine, or halo-lower alkyl, such as trifluoromethyl; cyclopentanol, R-, S- or R,S-1-hydroxyethylcyclohexane or the like.

Preferred cosubstrates for the reduction are secondary alcohols, such as isopropanol, 4-methyl-2-pentanol or further other C,C-di-(lower alkyl)-methanols. These are preferably present in the reaction mixture in an excess, for example compared to the ketone to be reduced, for example making up 50 % (related to the volume of the reaction mixture, v/v). The concentration of the ketone to be reduced preferably lies below an upper limit of 3 mol/l, preferably up to 2,6 mol/l or lower.

Ketones to be reduced are especially free ketones, compounds carrying at least a single carbonyl group such as mono-, di- or tri-ketones, or ketals thereof (such as especially di-(lower alkyl) ketals or (cyclic) (unsubstituted or substituted, for example by lower alkyl, such as methyl) lower alkylene ketals, such as ethylene ketals (dioxolan derivatives).

Examples are oxo-carrying isoprene derivatives, such as mono-, di- or triterpenes, e.g. menthone, pulegone, carvone, carone, verbenone, camphor, dihydrocarvone, dihydrocarvone hydrobromide, carvenone, hemanulcine or taxol or lanosterol, or steroids, such as androsterone, testosterone, estrone, cortisol, corticosterone, aldosterone or prednisone; di-(lower alkyl)- or lower alkyl-lower alkenyl-ketones, such as acetone, butan-2-one, n-hexan-2-one, n-heptan-2-one, n-octan-2-one, n-nonan-2-one, 3-octanone, 5-methyl-2-heptanone, 3-octen-2-one, 3-penten-2-one, 6-methyl-hex-5-en-2-one or 6-methyl-hept-5-

en-2-one; hydroxyacetone; or n-decan-2-one; acetophenone, methyl-1-(1- or 2-naphthyl)-ketone, methyl-1-phenylmethyl-ketone, 1-phenyl-2-propanone, benzophenone or phenacyl halogenides, wherein the naphthyl- or phenyl moieties in the moieties mentioned are in each case unsubstituted or substituted by one or more, preferably one to five substituents selected independently of each from the group consisting of lower alkoxy, such as methoxy, lower alkyl, such as methyl, nitro, cyano, halogen, such as fluorine, chlorine, bromine or iodine, or halo-lower alkyl, such as trifluoromethyl; 2-furylethanone; cyclopentanone, cyclohexanone, 2-methylcyclohexanone, cycloheptanone, methyl-cyclohexyl-ketone; keto esters, such as methylpyruvate, ethylpyruvate, ethyl-bromopyruvate, ethyl-3-methyl-2-oxobutyrate, ethyl-3-oxo-butyrate, methyl-3-oxobutyrate, phenylglyoxylic acid methylester, phenylglyoxalic acid ethylester, 2-oxo-4-phenylbutyric acid methylester or the corresponding tert-butyl- or neopentyl ester.

For the use according to the invention in the oxidation or reduction in the presence of the biocatalyst according to the invention, customary conditions for chemical reactions of that type are chosen.

The preferred pH range for the oxidation of alcohols in the presence of ketones as co-substrates is kept in the area from pH 6 to pH 12, more preferably from pH 8 to pH 11. For the reduction, the preferred pH range lies between pH 5 and pH 9, more preferably between pH 6 and pH 8. The pH value is controlled by standard buffers, for example phosphate buffers with alkaline metal, especially potassium or sodium phosphate buffer, boronic acid/HCl/sodium hydroxide buffers, or other buffers, such as Tris/HCl, HEPES buffers or the like; and/or by automated titration with an acid, such as HCl, to keep the pH from rising, or a base, such as NaOH, to keep the pH from sinking.

The amount of the enzyme used is adapted to the amount of the substrate to be converted and can be calculated conveniently from the activity of the biocatalyst according to the invention (see e.g. purification table = Table 3 in Example 2).

The enzyme of the invention can also be used in the presence of surface active substances (surfactants, detergents). As detergents, for example,

- anionic tensides, that usually include long chain fatty acids as anionic, hydrophobic component, e.g. sulfates of long chain (especially C<sub>8</sub>-C<sub>18</sub>) alcohols, such as alkaline metal C<sub>8</sub>-C<sub>18</sub>alkanoylsulfates, especially sodium dodecylsulfate or sodium decylsulfate;
- cationic tensides, which besides hydrophobic, aliphatic or aromatic (especially alkyl) moieties include hydrophilic groups with a positive charge (e.g. quaternary ammonium), such as benzyl-dimethylstearylammmonium chloride or cetylpyridinium chloride;
- amphoteric detergents, such as mono- or dicarboxylated imidazolines of fatty acids, such as sodium lauryldicarboxyimidazoline or sodium; or
- non-ionic tensides, such as ethoxylated sugar esters of higher fatty acids, such as polyoxyethylene-sorbitan-monolaurate, -palmitate, -stearate or -tristearate.

The temperatures for the use of the enzyme of the invention in the oxidation or reduction reactions preferably lies in the range customary for biocatalytic reactions or above, preferably in the range from 10 to 65, more preferably from 40 to 65 °C.

A preferred embodiment of the use according to the invention is in the stereoselective (enantioselective or, in the presence of more than one center or axis of asymmetry, diastereoselective) production of chiral (or diastereomeric) alcohols from the corresponding ketones (especially of the formula II wherein R<sub>1</sub> and R<sub>2</sub> have different meanings) or form an asymmetric (e.g. asymmetrically substituted) bridge with prochiral oxo-carrying carbon by reduction of the prochiral C=O group. The oxidation of or reduction to the S-enantiomer is especially preferred in this reaction. Enantiomeric purities in excess of 90 %, especially of 96 % (in terms of the enantiomeric excess = ee = 100 (2x - 1) where x is the molar fraction of the prevailing enantiomer) or more, most preferably of 99 % or more, are obtained with the enzyme of the invention.

Yet another preferred embodiment of the invention relates to the use of/process using the enzyme of the invention for the (mild) chemoselective (especially stereoselective) oxidation of secondary alcohols, where only the hydroxy group with the appropriate steric form is oxidised to the corresponding oxo group (while other oxidable hydroxy groups in the same or other molecules remain intact). This can especially be used for the separation of isomers where from mixtures of alcohols only the reactive ones are oxidised, so that either the desired alcohols remain as such or the resulting oxo compounds are, in a subsequent step, again transformed into the desired alcohols by reversal of the reaction (reduction).

An especially preferred variant of this use (process) relates to the use of an enzyme according to the invention for the (mild) enantioselective oxidation of only one isomer of mixtures of enantiomers (or diastereomers), especially racemates, of secondary alcohols, especially of alcohols of the formula I wherein  $R_1$  and  $R_2$  are two different moieties, especially as defined above, or form an asymmetric (especially asymmetrically substituted) bridge. By this method, the remaining alcohol can be obtained in isomerically pure, especially enantiomerically pure, form, e.g. with 75 % or more, especially 95 % or more, more especially with 98 % or more enantiomeric excess regarding the carbon atom carrying  $R_1$  and  $R_2$ .

The process of the invention can thus be used especially for separating mixtures of stereoisomers with respect to a center of chirality by kinetic resolution, if one stereoisomer of an alcohol is specifically oxidized, or for the stereospecific production of secondary alcohols representing a specific chiral form from ketones.

Still another preferred embodiment of the invention relates to the use of/ a process using an enzyme of the invention for the mild chemoselective reduction of ketones where only specific oxo groups are converted into the corresponding secondary hydroxy group(s), while other reducible groups (less amenable keto groups, C=C double bonds, nitro groups or the like) remain intact. This can also be used for separation processes in that from complex mixtures of ketones only the reactive ones are taking part in the reaction while either the desired oxo compounds remain in the reaction mixtures or the resulting alcohols are, in a subsequent inverse step (oxidation), converted back into the desired keto compounds. As in that reaction prochiral oxo-substituted carbon atoms are transformed into the corresponding asymmetrically substituted hydroxy-carrying carbon atoms, this is also appropriate for obtaining the isomerically, especially enantiomerically, pure alcohols (especially with a purity as defined above).

The process of the invention can be performed with free or immobilized biocatalyst according to the invention, which can be used in enriched or preferably in purified form. In another embodiment of the invention a recombinant microorganism (especially a host cell) which is present in suspension or immobilized and is expressing an enzyme of the invention is used for performing the reaction, i.e. the enzyme is in cell-bound form.

For example, an enzyme of the invention for use in a process of the invention may be immobilized. The immobilization of said enzyme can be carried out analogously to processes known *per se*, e.g. coupling to a solid support or enclosing in an enzyme membrane reactor.

The process of the invention may also be lead in the presence of a (free or immobilized) microorganism transformed by genetic engineering techniques with a gene coding for an enzyme of the invention to be able to produce the desired enzyme of the invention, especially in higher amounts than it would be present in the original microorganism, e.g. *Rhodococcus ruber*, especially *Rhodococcus ruber* DSM 14855.

The reaction of an alcohol or ketone substrate with an enzyme of the invention or a microbial cell extract is preferably carried out in homogeneous aqueous solution at pH 5 to 10.5, more preferably at pH 6 to 9.5. For the stabilization of the pH value, the reaction is carried out in a manner known *per se* in buffered solution or using a pH-stat. The reaction temperature is approximately from 10 to 65 °C, more preferably from 20 to 50 °C, even more preferably from 20 to 30 °C. The substrate is used preferably in a concentration of 1 mM to 2 M, more preferably 50mM to 500 mM. However, if the substrate is less soluble, it is also possible to use a substrate suspension.

The process according to the invention can be carried out either as a batch process or continuously in an enzyme membrane reactor (EMR). In the latter case, the enzyme membrane reactor is preferably fitted with an ultrafiltration membrane having a separation limit of less than approximately 30 000 Da, so that the enzymes contained in the reaction mixture are held back whilst the low-molecular-weight products and unreacted reactants pass through the membrane and the product can be isolated from the outflow. The reactor is preferably sterilized before use so that the addition of antibacterial substances can be dispensed with. The reactions are carried out in a manner analogous to that described above.

The process according to the invention can also be carried out by percolating the solution containing the alcohol or ketone substrate, which has been adjusted to a suitable pH value, through a solid carrier on which the enzyme of the invention has been immobilized (the

matrix-bound enzyme preparation is obtainable, for example, by percolation of the crude microbial extract through CNBr-activated Sepharose, Eupergit or the like).

Working up the reaction mixture and purification of the products in accordance with the invention are carried out by customary processes known from the State of the Art. For example, the reaction mixture can be clarified by filtration or, preferably, centrifugation, and then the enzyme can be separated by ultrafiltration (membrane with separation limit of  $\leq 30$  kDa) and the remaining product can be washed out of the retentate by diafiltration.

The isolation of the desired products obtained according to any of the variants of the use of or process using the enzyme of the invention (secondary alcohol or ketone) is achieved using standard methods, such as distillation or rectification (fractionated distillation), steam distillation or azeotropic distillation, or using chromatographic methods, just to recite some examples to which the skilled person may add other methods as convenient or necessary.

Salts of educts or products can be converted into the free compounds, free compounds into the salts using standard methods, respectively.

Nucleic acids are preferably DNA or RNA (in general, oligo- or polynucleotides).

Isolated nucleic acids coding for a biocatalyst according to the invention with alcohol dehydrogenase activity, especially recombinant nucleic acids, are preferably obtained and defined as follows:

A nucleic acid, especially a gene, coding for a biocatalyst of the invention can, for example, be obtained by identifying at least a part of the sequence of an isolated enzyme of the invention, deducing DNA sequences coding for the partial protein sequence, preparing an oligonucleotide or a mixture of oligonucleotides (taking into consideration the degeneracy of the genetic code) as probe(s), probing a DNA library derived from the microbial strain naturally expressing the activity of the biocatalyst (the term DNA library also including a "cDNA-library"), isolating the gene, and cloning it into a suitable vector for transformation of the microorganism to be genetically modified. All these methods (especially those depicted below for identification of, transformation with and expression of nucleic acids coding for an enzyme of the invention) are standard practice, e.g. as described in Sambrook et al.,



Molecular Cloning – A Laboratory Manual, 2<sup>nd</sup> edition, Cold Spring Harbor Press, 1989, or in Gassen et al., "Gentechnische Methoden – Eine Sammlung von Arbeitsanleitungen für das molekularbiologische Labor", Spektrum Akademischer Verlag, Heidelberg 1999, in F.M. Asubel (Hg.) "Short Protocols in Molecular Biology", 3<sup>rd</sup> ed., New York, Wiley 1997; or in Asubel et al., "Current Protocols in Molecular Biology", Vol. 1-3, Greene Publishing Associates and Wiley-Interscience, New York, 1987.

The partial sequencing of an enzyme of the invention is, for example, made using selective endoproteases for selective digestion, e.g. endo-protease Lys-C, endoprotease Glu-C, chymotrypsin, thermolysin or preferably trypsin (cleaving C-terminally from the basic amino acids arginine or lysine) and, after separation, e.g. electrophoretically on a gel or by chromatography (e.g. HPLC), determining the terminal sequences of the resulting peptides, e.g. by exopeptidases, e.g. carboxypeptidases, such as carboxypeptidase A, B or P). Preferred is tryptic digestion, then MS/MS analysis (TOF).

DNA libraries can also be obtained by PCR methods.

A cDNA library (obtainable e.g. after extraction of the mRNA from the cells, transformation into DNA using reverse transcriptase, introduction of sticky ends, introduction into a cloning vector, and introduction of that vector into an appropriate host cell, e.g. a plasmid vector into a bacterium, such as a bacteriophage  $\lambda$  vector or a cosmid into *E. coli*, a yeast artificial into a yeast, such as *Saccharomyces cerevisiae*, a *Pichia-pastoris* vector into *Pichia pastoris*, or the like) or a DNA library (e.g. obtainable from selective digests of isolated DNA with restriction endonucleases, especially of type II, e.g. Alu I, Bam HI, Bgl I, Bst I, Eco RI, Eco RII, Fok I, Fnu DI, Hae II, Hae III, Hind II, Hind III, Hpa I, Msp I, Not I, Pst I, Sac I, Sal I, Sau 3A, Sma I, Taq I, Xho I, Xma I; if necessary, filling up recessed termini with Klenow fragment of *E. coli* DNA polymerase I, then ligating, e.g. with bacteriophage T4 DNA ligase, into a bacteriophage  $\lambda$  or cosmid vector for expression in *E. coli* or into a yeast artificial chromosome vector for expression in yeast) of the genomic DNA of the microorganism from which the enzyme of the invention can be isolated is then screened by stringent hybridization for matching polynucleotides (for appropriate conditions for stringent hybridisation see, for example, Sambrook et al., loc. cit., chapter 9) using (radioactive or fluorescence labeled) probes deduced from the known peptide fragments based on the genetic code which are produced according to standard procedures (fully degenerate, partially degenerate or using

"guessmers"), which polynucleotides can then be isolated (e.g. from agar gels or the like) and sequenced. Longer nucleotides can be separated e.g. using pulsed field electrophoresis.

Hybridization is done using standard procedures, if necessary removing possible disturbing non-coding sequences, e.g. by PCR amplifying only the desired sequence parts or by endonuclease digestion, e.g. using dot blots of colonies of microorganisms from the DNA library. The positive clones can then be isolated.

The sequencing is done using standard procedures, e.g. the Maxam-Gilbert or the Sanger method.

If necessary, by combination of overlapping partial sequences the complete sequence coding for an enzyme of the invention (or one subunit thereof, if more than one polypeptide form the complete enzyme) can be determined.

From this sequence, the corresponding amino acid sequence of the enzyme is (or, if more than one polypeptide forms it, the subunits thereof, the amino acid sequences are) easily determined, using the genetic code.

In another approach, the full amino acid sequence of the isolated biocatalyst according to the invention can be determined (e.g. by different endopeptidase digests and matching of overlapping sequenced partial peptides) and a DNA coding the protein can be produced synthetically. It is also very easily possible to screen a suitable DNA library in a host, e.g. *E. coli*, for expression of temperature resistant alcohol dehydrogenase activity to obtain a transformed clone expressing the biocatalyst. Still another method makes use of antibodies against an enzyme of the invention that can be obtained using standard procedures (up to and including the production of monoclonal antibodies obtained from myelomas obtained according to standard procedures) in order to isolate the ribosomes carrying the mRNA coding for the enzyme, transforming it into the corresponding DNA (e.g. with reverse transcriptase) and sequencing or genetically engineering the resulting enzyme.

The nucleic acid according to the invention is preferably present in isolated form or in recombinant form (then also in a microorganism, see below).

The nucleic acids according to the invention also comprise modified (especially recombinant, but also naturally occurring) nucleic acids where, when compared with the form sequenced as described above, one or more nucleic acids are deleted, inserted, exchanged or added terminally, as long as the polypeptide or polypeptides for which they code still display alcohol dehydrogenase activity, especially according to the test method with 1-phenylethanol or acetophenone as described in the Examples.

Terminal additions may comprise the addition of sequences for vectors or host nucleic acids into which the coding sequence may be combined.

More preferably, the modified nucleic acids are modified such as to code for a biocatalyst with alcohol dehydrogenase activity, obtained by recombinant technology (resulting in a recombinant nucleic acid) or alternatively from natural sources, where the amino acid sequence of the biocatalyst comprises deletions, insertions, terminal additions or exchanges (especially conservative exchanges, e.g. of lipophilic against lipophilic, basic against basic, acidic against acidic, polar against polar amino acids, or the like) of amino acids (preferably of up to 20, in case of terminal additions up to 1000; more preferably of up to 5, in case of terminal additions of up to 200 amino acids, respectively), or any combination of such changes, when compared to the sequence of the enzyme as purified in the examples or possible different subunits thereof, as long as the basic activity (alcohol dehydrogenase activity, especially with the substrates (preferably 1-phenylethanol or acetophenone) and co-substrates mentioned in the examples) is still present, especially in connection with one or more of the additional advantageous properties mentioned for a biocatalyst according to the invention.

Most preferably, the modified nucleic acids contain 1 to 50, more preferably 1 to 12, additional nucleotides by insertion (especially additions yielding no frame shift), 1 to 50, more preferably 1 to 12 changes in nucleic acids, preferably resulting in conservative amino acid changes, and/or 1 to 50, more preferably 1 to 12, deletions of nucleotides, especially without frame shift.

The invention also relates to probes, especially in radiolabelled or fluorescence labelled form, that are hybridizable under stringent conditions to genomic or cDNA or other nucleic

acids coding and that code for the sequences of the six partial amino acid sequences given as SEQ ID NO: 1 to SEQ ID NO: 6, or for parts thereof, said probes preferably having a length of 6 to 24, more preferably of 12 to 21 nucleotides.

The embodiment of the invention relating to microorganisms transformed with a nucleic acid coding for such a biocatalyst with alcohol dehydrogenase activity preferably relates to microorganisms appropriate for expressing the gene, but also those that comprise the nucleic acid for pure conservation or replication purposes.

Appropriate microorganisms are especially viruses, bacteriophages or especially host cells, for example, bacteria, e.g. *E. coli*, single cell fungi, such as yeasts, e.g. *Pichia pastoris*, *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae*, or plant cells.

The microorganisms, especially host cells, can be transformed with nucleic acids as such that code for an enzyme of the invention; however, usually they are transformed with suitable vectors, e.g. plasmids, cosmids, yeast artificial chromosome or the like, which may comprise partial sequences (e.g. useful in sequence determination) or total sequences for the enzyme (e.g. useful in the expression of the enzyme).

Transformation of host cells is made according to standard procedures known in the art and appropriate for the respective host cells, e.g. according to the calcium chloride method, by electroporation, transformation after spheroblast formation into fungi, transformation with polyethylene glycol, transformation with lithium chloride, or the like. Virus or the like are modified by introduction of the sequences comprising the coding sequences for the enzyme of the invention.

Especially, the invention relates to the use of the microorganisms, especially host cells, in the production of said biocatalyst with alcohol dehydrogenase activity.

Expression systems suitable for production of an enzyme of the invention are especially phage-based expression systems in bacteria, e.g. bacteriophage  $\lambda$  or cosmids for *E. coli* as host, yeast artificial chromosomes for expression in *Saccharomyces cerevisiae*, the *Pichia pastoris* expression system used for expression in *Pichia pastoris*, expression systems in *Schizosaccharomyces pombe*, the baculovirus expression system or the like. In each of these

systems, the nucleotide sequences coding for an enzyme of the invention can be expressed, either as 'such or' with additional N- or C-terminal sequences, e.g. such that allow for direct export of the resulting polypeptide outside the expressing cells. These extra sequences, if disturbing the activity or otherwise not desired, can then be cleaved off using appropriate endoproteases known in the art.

#### Preferred embodiments of the invention

Preferred embodiments of the invention are represented in the dependent claims, where in each case more general terms can be replaced with more specific terms provided in the definitions above, independently of other terms, in order to define still more preferred embodiments.

The invention relates in particular to the use of/process using the enzyme of the invention, and especially to the enzyme described in the Examples.

The following examples are illustrative, however, do not limit the scope of the present invention.

#### Examples:

##### Example 1: Cultivation of *Rhodococcus ruber* DSM 14855

The Gram-positive bacterium *Rhodococcus ruber* DSM 14855 is grown under aerobic conditions in baffled Erlenmeyer flasks at 30 °C and 130 rpm using a complex medium containing yeast extract, peptone, glucose and mineral salts (10 g/l yeast extract (OXOID CM129, OXOID Ltd., Hampshire, England), 10 g/l peptone, 2 g/l NaCl, 0.15 g/l MgSO<sub>4</sub> • 7H<sub>2</sub>O, 1.3 g/l NaH<sub>2</sub>PO<sub>4</sub>, 4.4 g/l K<sub>2</sub>HPO<sub>4</sub>) for three days. Cell growth is followed by determination of the optical density via absorption at 546 nm, see Table 1. After a centrifugation (2000 g, 20 min), the pellet is taken up in Tris-HCl buffer pH 7.5 (50 mM), shock-frozen in liquid nitrogen and lyophilised.

The cells produce several NAD<sup>+</sup>/NADH-dependent sec-alcohol dehydrogenases without any induction. The highest sec-alcohol dehydrogenase activity is displayed during the late exponential and early stationary phase of the growth curve (see table 1). The activity is measured as the ability of the cells to oxidise 1-phenylethanol or the ability to reduce acetophenone.

For this test, whole lyophilised cells of *Rhodococcus ruber* DSM 14855 (20 mg) are rehydrated in phosphate buffer (0.5 ml, 50 mM, pH 7.5) for 30 min. Activity is measured by adding acetophenone (0.27 mmol) and 2-propanol (0.4 ml). The mixture is shaken at 24 °C, 130 rpm in eppendorf vials for 2.5 h. The reaction is quenched by addition of ethyl acetate (1 ml) and centrifugation. The conversion is determined by GC (Varian 3800, FID) on an achiral column (HP-1301, 30 m x 0.25 mm x 0.25 µm; N<sub>2</sub>). Temperature program for *rac*-1-phenylethanol/acetophenone: start temperature 80 °C – hold 2 min – 10 °C/min – until 130 °C – hold 2 min. The conversion is calculated from calibration curves. For the inverse test, 1-phenylethanol is oxidised in the presence of acetone as co-substrate.

**Table 1: sec-Alcohol dehydrogenase activity versus growth curve for *Rhodococcus ruber* DSM 14855**

Relative Cell Density (OD 546 nm)	Time of growth (h)	Sec-alcohol dehydrogenase activity (%)
3.7	0	35
2.8	2	34
2.7	4	37
3.4	6	59
3.6	8	65
4.8	10	81
6.5	12	86
7.2	14	78
9.1	16	81
10.1	18	84
11.0	20	100
12.4	22	66
15.9	24	54
21.0	26	42
27.2	28	46
34.4	30	34
39.7	32	43

38.1	34	34
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**Example 2: Purification of a sec-Alcohol dehydrogenase 'ADH-A' from *Rhodococcus ruber* DSM 14855**

**a) Cell Disruption**

Bearing in mind that the sec-alcohol dehydrogenase might be a membrane-associated or even membrane-bound protein, cell disruption using a Vibrogen cell mill (glass beads, diameter 0.25 mm, Vibrogen Zellmühle, E. Bühler, Typ VI-4; Braun Biotech Int., Melsungen, Germany) is used and optimised in order to obtain the majority of the activity in the cell-free lysate and not in the cell debris fraction. This results in an exceptionally long and rough procedure of 7 shaking cycles of 2 min agitation/ 5 min cooling each. The enzyme shows high stability against this strong mechanical treatment. The buffer used for cell disruption is 10 mM Tris-HCl buffer, pH 7.5.

**Table 2: Optimization of the cell disruption by increasing number of shaking cycles**

Relative Activity [%]	48	60	71	85	82	97	100	93	82
Number of shaking cycles	1	2	3	4	5	6	7	8	9

**b) Pre-Treatment with DEAE-Cellulose:**

Due to the harsh breaking of the cells, several undesired materials such as carotenoids, lipids and surface-active components are released from the cells beyond the sec-alcohol dehydrogenase activity. Those components lead to immense filtration problems and are thus removed by treatment with DEAE (Diethylaminoethan)-Cellulose (Sigma) in a batch procedure. All sec-alcohol dehydrogenase activity is bound onto the matrix in 10 mM Tris-HCl buffer pH 7.5 and can be eluted by addition of 0.5 M NaCl to the buffer.

### c) Purification Steps

The purification of the *sec*-alcohol dehydrogenase by FPLC (Fast Protein Liquid Chromatography)-methods starts with the cell-free lysate after pre-treatment with DEAE-Cellulose.

#### (i) Hydrophobic Interaction Chromatography

The first protein purification step is a Hydrophobic Interaction Chromatography using Phenyl Sepharose High Performance material ((Amersham Pharmacia Biotech AB, Uppsala, Sweden). During sample preparation precipitation occurs by adding  $(\text{NH}_4)_2\text{SO}_4$ , and solids are removed since they do not contain any *sec*-alcohol dehydrogenase activity. With addition of  $(\text{NH}_4)_2\text{SO}_4$  as salting-out medium to the equilibration buffer and a stepwise gradient, the different hydrophobicities of proteins enable a first separation of enzymes. Two  $\text{NAD}^+/\text{NADH}$  dependent *sec*-alcohol dehydrogenase activities are found, but just one fraction, arbitrarily called *sec*-ADH-A' (standing for *sec*-alcohol dehydrogenase + 'acetone') shows stability against the co-substrates acetone/2-propanol. The second activity, called B<sub>1</sub>, is significantly less stable and is thus not further investigated.

Details of step (i): Column length 75 mm, diameter 16 mm. Eluent: A: B + 1 M  $(\text{NH}_4)_2\text{SO}_4$ ; B = 10 mM Tris-HCl buffer pH 7.5.

Application volume: 3 times 46 ml are administered (3 separate runs).

Elution: first 70 ml A, then 47,5 ml 50 % A + 50 % B, then 47,5 ml 25 % A + 75 % B, then linear gradient during 47,5 ml from 25 % A + 75 % B to 100 % B and further elution with this eluent. Flow rate 4 ml/min.

Activity of B<sub>1</sub> is found between 70 and 118 ml. Activity of *sec*-ADH-A' is found between 220 and 236 ml elution volume. The latter (3 x 16 ml after 3 runs, a total of 48 ml) is used for further purification.

#### (ii) Ion Exchange Chromatography

The complete fraction (46 ml) containing *sec*-ADH-A' is further purified by Ion Exchange Chromatography using the anion exchanging column UNO Q6 (BioRad). By running an optimised stepwise gradient using chloride as counter ion in the elution buffer, the *sec*-



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alcohol dehydrogenase activity is separated again into two  $\text{NAD}^+/\text{NADH}$  dependent fractions, whereof again one is identified as *sec*-ADH A. The second *sec*-ADH B<sub>2</sub> is significantly less stable and thus is not further investigated.

Details of step (ii) .Column length 53 mm, diameter 12 mm.

Elution buffer: A 10 mM Tris-HCl-buffer pH 7.5; B: A + 1.5 M NaCl ("100 % NaCl).

Gradient: First elution with 62 ml of A, then with 67 ml of 82,75 % A + 17,5 % B, then with 28 ml of 50 % A + 50 % B, finally with pure B. Flow rate 4 ml/min.

Activity of B<sub>3</sub> is found between 68 and 82 ml. Activity of *sec*-ADH-A' is found between 131 and 137 ml elution volume. The latter (6 ml) is used for further purification.

#### (iii) Affinity Chromatography

The third step in protein purification of the resulting 6 ml from step (iii) is carried out on an innovative material, Blue Sepharose Cl-6B [see Shaw et al., Biochem. J. 187, 181 (1996)], containing a dye, Cibacron Blue F3G-A, specific for enzymes requiring adenylyl-containing cofactors (such as  $\text{NAD}^+/\text{NADP}^+$ ) (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

In contrast to theory, *sec*-ADH-A' does not bind on the matrix but elutes right after the void volume, regardless of the conditions used. But at linear increased salt concentration, another *sec*-ADH is found and is identified again as  $\text{NAD}^+/\text{NADH}$  dependent *sec*-alcohol dehydrogenase B<sub>3</sub>, which is significantly less stable and thus not further investigated.

Details of step (iii): Column length 60 mm, diameter 16 mm.

Eluent: Buffer A: 10 mM Bis-tris-HCl buffer pH 6.0; Buffer B: A + 1 M NaCl.

Gradient: First 31 ml of buffer A, then linear gradient from 100 % buffer A to 100 % buffer B. Flow Rate 4 ml/min.

Activity of alcohol dehydrogenase B<sub>3</sub> is found between 44 and 68 ml. Activity of *sec*-ADH-A' is found between 7 and 23 ml elution volume. The latter is concentrated up on Centriplus YM-10, cut-off 10 kDa (regenerated cellulose with 10 kDa exclusion size) from Millipore (Millipore GmbH, Vienna, AT)/Amicon to a final volume of 2 ml.

#### (iv) 2.3.4 Size Exclusion

Size exclusion using this Superdex 200 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) leads to final purification, the only *sec*-alcohol dehydrogenase activity detected is *sec*-ADH A. 10 runs with 0.2 ml each of the resulting 2 ml in step (ii) are used.

As the column is calibrated with several standard proteins, namely yeast alcohol dehydrogenase (150 kDa), Bovine Serum Albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and blue dextran (2 kDa), the molecular weight of the native enzyme is determined as approximately 62 kDa.

Details for step (iv): Column length 310 mm, diameter 10 mm.

Eluent: 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.15 M NaCl, pH 7.0

Flow Rate 4 ml/min.

10 times 1 ml of fractions containing the activity of *sec*-ADH-A' are obtained (after about 13 ml of elution). The molecular weight is determined based on the peak concentration of the ADH-A activity.

Summary of the purification protocol:

Following this protocol, the *sec*-alcohol dehydrogenase 'ADH-A' is purified reproducibly. The small overall recovery is explained by a major loss of activity during desalting procedures and buffer exchange *via* dialysis and during concentration to a small volume before applying it to the size-exclusion column. For this reason, the majority of biochemical characterizations are carried out with *semi*-pure enzyme after the dye chromatography, where no other *sec*-alcohol dehydrogenase activity is left.

In addition, the "low" yield is attributable to the fact that in the cell extract at the beginning other alcohol dehydrogenases that are removed during the later procedure (e.g. B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>) contribute to the dehydrogenase activity.

In the following table, 1 U = 1 µMol/min.

The activity is determined by oxidation of 1-phenylethanol (6.6 µM) and addition of 10 mM NAD<sup>+</sup> (testing conditions: 30 °C, 10 µM Tris-buffer, pH 7.5, 10 min, conversion by GC-

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analysis). Protein amounts are measured by the method of Bradford (Coomassie Blue Protein Assay) at 595 nm using a BioRad Protein Assay:

**Table 3: Purification Table**

Step	V [ml]	Protein [mg/ml]	Tot. Protein [mg]	Activity [Units/ml]	Tot. Act. [Units]	Spec. Act. [Units/mg]	f	Yield [%]
crude extract	122	8,2	1000	0,13	16	0,016	1	100
DEAE Cell.	139	3,4	472	0,27	37	0,078	5	232
Ph. Sepharose HP	48	1,4	67	0,17	8	0,123	8	52
UNO Q6*	6	0,7	4,2	0,47	3	0,672	43	18
Blue Sepharose	16	0,03	0,5	0,07	1	2,2	138	7
Superdex 200*	10	0,0075	0,075	0,13	1,3	17,5	1100	8

**Example 3: Purification control and molecular weight determination by gel electrophoresis:**

The progress of the protein purification is controlled by native as well as by SDS-polyacrylamide gel electrophoresis (PAGE). For both methods, the protein fractions are subjected to a Laemmli SDS-PAGE system using a MINI-PROTEAN II dual slab cell (BioRad).

**a) Native Gel Electrophoresis**

Active fractions are treated with non-denaturing sample buffer and loaded on a polyacrylamide gel (12%) without SDS. The gel is run at 4 °C at 150 V with running buffer containing 15 g/l Tris and 72 g/l Glycine. The method for visualization of *sec*-alcohol dehydrogenases in polyacrylamide gels was first reported by Grell et al. [see Science **149**, 80 (1965)] and is based on a staining solution [see Dodgson et al., Biochem. J. **187**, 703 (1996)] mixed from aqueous 4 mM 2-octanol (or any aliphatic, long-chained *sec*-alcohol accepted as substrate) and 100 mM Tris-HCl buffer pH 8.5 containing 3 g/l NAD<sup>+</sup>, 0.1 g/l phenazinemetosulphate (N-methyldibenzopyrazine ethylsulfate salt) and 1 g/l nitro blue tetrazolium. Purple spots of the reduced formazane are usually visible within 30 min of incubation and display the presence of a *sec*-alcohol dehydrogenase. For result see Fig. 1.

There are at least seven different *sec*-alcohol dehydrogenases present in the microorganism, of which *sec*-alcohol dehydrogenase 'ADH-A' can be separated properly during the purification protocol.

**b) SDS polyacrylamide gel electrophoresis**

In this case, the same protein samples used for native electrophoresis are denaturated with sample buffer (SDS reducing buffer, BioRad) and administered to a SDS-polyacrylamide gel (12%) subsequently run at 200V at room temperature in the same running buffer with addition of 3 g/l SDS. After the run, staining is carried out with Coomassie Brilliant Blue. The molecular weight is determined in comparison to a low molecular weight range SigmaMarker as protein standard containing albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa),  $\alpha$ -lactalbumin (14.2 kDa) and aprotinin (6.5 kDa). The result is a single band after the size exclusion corresponding to 38 kDa.

**Example 4: Characterisation of *sec*-alcohol dehydrogenase "ADH-A" from *Rhodococcus ruber* DSM 14855**

**a) Determination of Molecular Weight:**

The molecular weight is determined by running SDS-polyacrylamide gel electrophoresis and in parallel by a size exclusion column calibrated with standard proteins. The elution of the purified protein on Superdex 200 indicates a molecular mass for the native enzyme of about 62 kDa. In contrast, the SDS-PAGE procedure results in a single band at 38 kDa. This discrepancy may be explained by the structure of the protein, as either (i) long thin proteins elute generally earlier than globular proteins or (ii) a dimer may be present. Due to the unsymmetric peak form of the protein in relation to the standards, the determination of the peak maximum limits the accuracy of molecular weight calculations.

**b) pH-Optimum**

The measurements are carried out in 25 mM Tris-maleate pH 6.0 – 9.0 and 50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 10.0 – 11.0, and the activity is determined *via* conversion of 1-phenylethanol or acetophenone (6.6 mM) by addition of 10 mM NAD<sup>+</sup> or NADH, respectively. The reaction time is 10 min at 50 °C. The present enzyme displays activity over a broad range of pH

values (see Table 4). The pH-optimum is different for the two possible reactions. While the enantioselective (ee >99%) reduction of ketones prefers a pH of 6.5 – 7.5, the oxidation of *sec*-alcohols is enhanced in more basic conditions and is very efficient over an extremely broad pH-range. The selectivity of the oxidation does not change when the pH is raised, i.e. ee = >98% at pH 9.0. Routinely, all reactions are conducted at pH 7.5 for both oxidation and reduction to minimize buffer exchange procedures.

2. Table 4: pH-optimum

Relative activity [%]	Oxidation	31	46	51	67	100	82	78
	Reduction	70	100	85	78	56	19	15
PH		6	6.5	7	8	9	10	11

c) Temperature Optimum

The effect of temperature on the *sec*-alcohol dehydrogenase 'ADH-A' is measured in 10 mM Tris-HCl buffer pH 7.5, 1-phenylethanol or acetophenone (6.6mM) are used as substrates in presence of 10 mM NAD<sup>+</sup>/NADH, respectively. Substrate and enzyme are incubated for 5 min using a thermostated shaker at a set temperature. Then the reaction is started at this temperature by addition of NAD<sup>+</sup>/NADH and the reaction is carried out for 10 min. Surprisingly, the *sec*-alcohol dehydrogenase 'ADH-A' shows maximum of activity within the range of 45 to 65 °C, an exceptionally high value (See Table 5). Inactivation occurs quite suddenly at 70 °C.

Table 5: Temperature Optimum

Relative Activity [%]	Oxidation	22	39	70	66	98	100	96	2
	Reduction	37	67	57	51	72	100	94	3
Temperature [°C]		12	20	29	38	46	54	64	70

d) Temperature Stability

For the determination of enzyme stability at different temperatures, oxidation as well as reduction are performed as described before in 10 mM Tris-HCl buffer pH 7.5 and within a temperature range from 25 to 50 °C. After incubation of the enzyme solution at 50 °C, the reaction is started by addition of the corresponding cofactor and is run for 10 min. Again (see Table 6), the *sec*-alcohol dehydrogenase 'ADH-A' displays an exceptional thermo-stability for a protein from *Rhodococcus ruber* DSM 14855, which is not known as a thermophile. The loss of activity after 24 hours is rather small for both the oxidation and reduction system, which indicates an enormous stability of the protein. Due to this property, a wide scope of industrial applications can be anticipated.

3. Table 6: Temperature stability

Relative Activity [%]	Oxidation 50 °C	100	94	77	8
	Reduction 50 °C	100	78	79	5
	Oxidation 30 °C	100	91	91	12
	Reduction 30 °C	100	75	73	6
Time [h]		0	8	24	48

d) Presence of Zinc

ICP-MS analysis shows the presence of Zn<sup>2+</sup> in the purified enzyme preparation (see Hemmers, B., et al., J. Biol. Chem. 275, 35786-35791 (2000), for the method). Whether this is required for catalytic activity and forms a true component of the enzyme is not determined at present. As the enzyme is both active in the presence of complex forming agents such as EDTA and in their absence, either the zinc is bound very firmly or it might not form part of the enzyme or its active site.

Example 5: Coupled Enzymatic Reduction/Oxidation System

Despite impressive progress in the regeneration of nicotinamide cofactors on the lab-scale using the 'coupled-enzyme method' [see, e.g., Hummel, Trends Biotechnol. 17, 487 (1999) or Chenault et al., Appl. Biochem. Biotechnol. 14, 147 (1987)], purified redox enzymes are not ideally suited for industrial applications due to their limited operational lifetime. In contrast, the so-called 'coupled-substrate-approach' is restricted due to the limited tolerance

of most ADH's towards elevated (co)substrate concentrations [see Faber, Biotransformations in Organic Chemistry, 4<sup>th</sup> edition, Springer, Heidelberg 2000, pp. 177-183]. In contrast, ADH-A is ideally suited for this simple protocol, which is fully commensurate with the requirements of industrial applications. The purified enzyme is exceptionally stable towards acetone or 2-propanol in up to 10% v/v concentration for cofactor-recycling in oxidation and reduction, respectively. In addition, excellent storage stability (no loss of activity after 14 days at +4 °C, or after several months at -80 °C, or in lyophilised form) makes this enzyme a prime candidate for large-scale applications. In particular, more lipophilic substrates are rapidly transformed due to their enhanced solubility in aqueous/organic systems (as compared to pure aqueous systems).

The following Tables show the reduction of various ketone substrates (Table 7) and the oxidation of various sec-alcohol substrates (Table 8).

The steric preference of the reduction follows the Prelog rules. In the case of oxidation, that enantiomer is oxidised that would be formed during reduction of the respective ketone.

Table 7: Reduction Of 2-methyl-2-hepten-6-one by partially purified enzyme (after Hydrophobic Interaction Chromatography), using 2-propanol as co-substrate (10  $\mu$ M substrate, 10 mM NADH, 30 °C; 100 % relative activity. 0.44  $\mu$ mol conversion/mg protein):

2-Propanol (% v/v)	Relative Activity (%)	ee
0	15	90
5	31	>99
10	34	>99
20	37	>99
30	44	>99
40	51	>99
50	61	>99
60	76	>99
70	100	>99
80	95	>99
90	25	>99



**Table 8:** Oxidation of 2-octanol by partially purified enzyme (after Hydrophobic Interaction Chromatography) using acetone as co-substrate (10  $\mu$ M substrate, 10 mM NAD<sup>+</sup>, 30 °C, 12h; 100% rel. act. = 13  $\mu$ mol conversion/mg protein):

Acetone (% v/v)	Relative Activity (%)	ee	E*
0	17	17	10
5	98	81	2
10	100	97	3
20	89	>99	10
30	88	>99	18
40	88	>99	58
50	97	94	100
60	62	36	>100
70	34	14	>100
80	15	5	>100
90	17	3	>100

\*) E is the Enantiomeric ratio (see Chen., C.-S., et al., J. Am. Chem. Soc. 104, 7294-99 (1982) (quotient of the reaction velocities of the isomers)

Analogously, the following reactions can take place:

Reduction of: 2-heptanone, 2-octanone, 2-nonanone, 2-decanone, 1-cyclohexylethanone, 1-(naphtha-2-yl)ethanone, oct-3-en-2-one (leading to the S-enantiomers in more than 97 % ee).

Oxidation of: rac-(CH<sub>3</sub>)<sub>2</sub>C=CH-(CH<sub>2</sub>)<sub>2</sub>-CHOH-CH<sub>3</sub>, rac-n-C<sub>6</sub>H<sub>13</sub>-CHOH-CH<sub>3</sub>, rac-4-phenyl-2-butanol, rac-(E)3-octen-2-on, cyclopentanol, rac-(1-(2-naphthyl)ethanol, rac-1-phenyl-1-ethanol.

Further, reduction of 2,5-hexandione yielding the the corresponding diol, and oxidation of a composition comprising 2R,5R-hexanediol and 2S,5S-hexanediol yielding the corresponding diketone.

**Example 6: Determination of Parts of the Protein Sequence of "ADH-A"**

Using a MS/MS de novo-sequencing protocol after tryptic digestion (see Lehmann, W.D., Massenspektroskopie in der Biochemie (= Mass Spectroscopy in Biochemistry), Spektrum Akad. Verlag, pp. 274-293, 1996 (ISBN 3-86025-094-9)) for the purified "ADH-A" enzyme obtained according to example 2, the following results are obtained:

4 tryptic peptides are sequenced completely (SEQ ID NO: 1-4), one of them also being found as part of a somewhat larger peptide (SEQ ID NO: 5) with possibly modified N-terminus, and a tryptic peptide with an ambiguity in the N-terminal sequence is found (SEQ ID NO: 6), where [L/I] is leucine or isoleucine and X is an unidentified amino acid:

Sequence	SEQ ID NO:
EVGADAAAR	1
TD[L/I]FEVVA[L/I]AR	2
SGAGAADA[L/I]R	3
V[L/I]AVD[L/I]DDDR	4
V[L/I]AVDXDDDXRX	5
[TD/DT] [L/I]MEVVA[L/I]AR	6 (either with TD in the beginning or with DT in the beginning)

**Example 7: Obtaining of nucleotide sequences corresponding to the enzyme "ADH-A"**

Taking one of the peptide sequences given above in Example 6 or two or more thereof, especially SEQ ID NO: 1, the corresponding (fully degenerate, then preferably only the nucleotides corresponding to 3 to 6 of the amino acids given are used; partially degenerate, then also longer nucleotides can be synthesized, with non-specific nucleotides at positions of ; or "guessmers" that have only one specific sequence) nucleotide sequence corresponding to (sense sequence) or complementary (antisense) to the nucleotide sequence of the corresponding mRNA, based on the standard genetic code, is synthesized (e.g. according to Narang, Tetrahedron 39, 3 (1986) or Itakura et al, Annu. Rev. Biochem. 53, 323 (1984); or using standard oligonucleotide synthesizers). For determination of useful oligonucleotides, their purification and use and the preparation of radio-labelled probes, the methods described by Sambrook et al., Molecular Cloning – a Laboratory Manual, Cold Spring Harbor Laboratory Press, pages 11.1 to 11.44 are be used. The labelled oligonucleotide or

oligonucleotides are then hybridised (for conditions see Sambrook et al., loc. cit., pages 11.45 to 11.61) to cDNA libraries (obtained according to Sambrook et al., loc. cit. pages 8.1 to 8.86). After identification of colonies with positive signals, these are grown and the vectors containing the hybridisable cDNA are isolated. The DNA is then sequenced according to standard methods (e.g. as shown in Sambrook et al., loc. cit., pages 13.1 to 13.103).

In one example, polymerase chain reaction is used for the preparation of the cDNA (see Lee, C.C., et al., Science 239(4845), 1288-91 (1988)).

The sequences between a start and a termination codon include the sequence(s) coding for the peptide backbone(s) of a part or the whole enzyme "ADH-A" or homologues displaying similar activity.

#### Deposition and Characteristics of the Microorganism

The following microorganism has been deposited on March 4, 2002, according to the Budapest Treaty with the DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig under the number DSM 14855:

*Rhodococcus ruber* DSM 44541 (now *Rhodococcus ruber* DSM 14855).

The strain has the following properties:

Colour RAL: 3012-3022 (salmon pink).

Morphology: elementary ramification rod/coccus growth cycle.

Biochemical properties: fatty acids: 5-30 % 16:0; 5-15 % 16:1; 5-15 % 18:0; 15-30 % 18:1; 15-30 % 18-Me. Mycolic acid C<sub>42-50</sub>.

Possesses epoxide-hydrolase, nitrilase and ester-hydrolase activities.

*Rhodococcus ruber* DSM 14855 is isolated from the lower Rhine on hexane as sole carbon source.

The strains can be kept on a complex culture medium as described above at 30 °C and 130 rpm in L-shaking flasks with flow spoiler. After centrifugation, the pellet can be taken up in

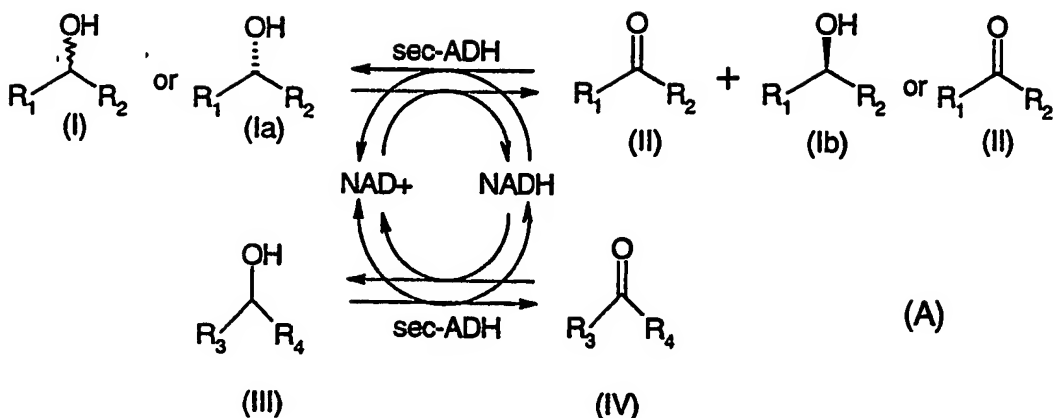
Tris-HCl buffer (pH 7.5, 50 mM), shock-frozen in liquid nitrogen and lyophilised, where desired and appropriate.

**Claims:**

1. A biocatalyst having alcohol dehydrogenase activity which can be obtained from *Rhodococcus*.
2. The biocatalyst according to claim 1, obtainable from *Rhodococcus ruber* DSM 14855
3. The biocatalyst according to claim 1 or 2 which shows stereospecific alcohol dehydrogenase activity in the oxidation of secondary alcohols or the reduction of ketones.
4. The biocatalyst according to any one of claims 1 to 3 in purified form.
5. The biocatalyst according to any one of claims 1 to 4 having a molecular weight on denaturing SDS polyacrylamide electrophoresis from 32 to 44 kDa, especially of about 38 kDa.
6. The biocatalyst according to any one of claims 1 to 5 having a molecular weight according to size exclusion chromatography of 55 to 69 kDa, especially of about 62 kDa.
7. The biocatalyst according to any one of claims 1 to 6 comprising  $Zn^{2+}$  bound to the molecule.
8. The biocatalyst according to any one of claims 1 to 7 including the partial sequence EVGADAAAR (SEQ ID No: 1) within the total sequence of at least one polypeptide forming the whole or part of the enzyme, or wherein one of the amino acids mentioned in the partial sequence is exchanged against a different amino acid.
9. The biocatalyst according to any one of claims 1 to 8 including the partial sequence TD[L/I]FEVVA[L/I]AR (SEQ ID NO: 2) within the total sequence of at least one polypeptide forming the whole or part of the enzyme, where [L/I] is leucine or isoleucine, or wherein one of the amino acids mentioned in the partial sequence is exchanged against a different amino acid.
10. The biocatalyst according to any one of claims 1 to 9 including the partial sequence SGAGAADA[L/I]R (SEQ ID NO: 3) within the total sequence of at least one polypeptide forming the whole or part of the enzyme, where [L/I] is leucine or isoleucine, or wherein

one of the amino acids mentioned in the partial sequence is exchanged against a different amino acid.

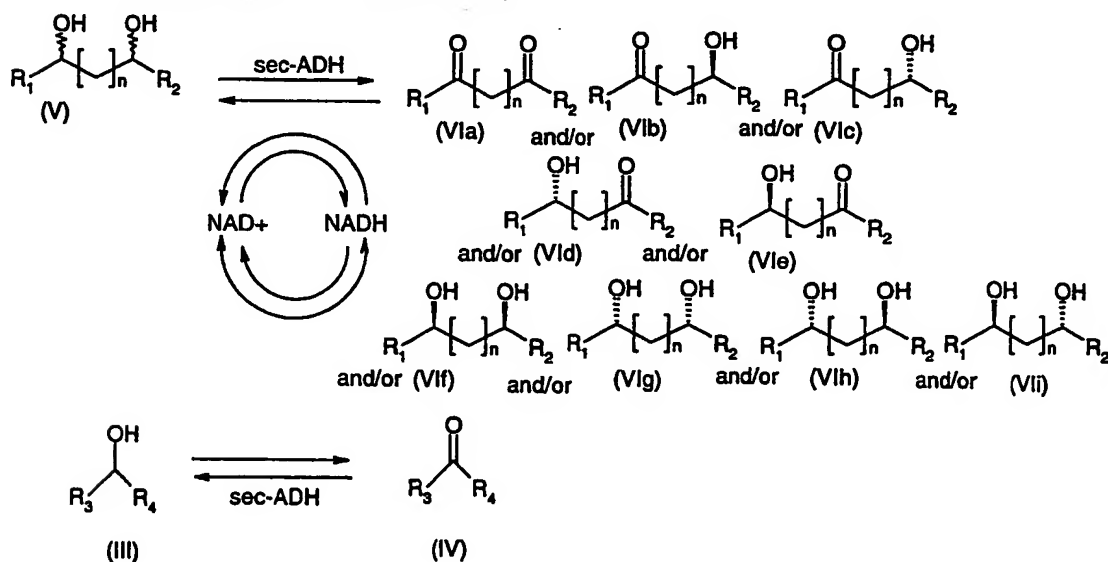
11. The biocatalyst according to any one of claims 1 to 10 including the partial sequence V[L/I]AVD[L/I]DDDE (SEQ ID NO: 4) within the total sequence of at least one polypeptide forming the whole or part of the enzyme, where [L/I] is leucine or isoleucine, or wherein one of the amino acids mentioned in the partial sequence is exchanged against a different amino acid.
12. The biocatalyst according to any one of claims 1 to 11 including the partial sequence V[L/I]AVD[L/I]DDDXRX? (SEQ ID NO: 5) within the total sequence of at least one polypeptide forming the whole or part of the enzyme, where [L/I] is leucine or isoleucine and X stands for an unidentified amino acid, or wherein one of the amino acids mentioned in the partial sequence is exchanged against a different amino acid..
13. The biocatalyst according to any one of claims 1 to 12 including the partial sequence [TD/DT] [L/I]MEVVA[L/I]AR (SEQ ID NO: 6, either with TD in the beginning or with DT in the beginning) within the total sequence of at least one polypeptide forming the whole or part of the enzyme where the sequence in brackets is selected from the two alternatives mentioned therein and where [L/I] is leucine or isoleucine, or wherein one of the amino acids mentioned in the partial sequence is exchanged against a different amino acid.
14. A recombinant biocatalyst according to any one of claims 1 to 13
15. A biocatalyst according to any one of claims 1 to 14, which shows alcohol dehydrogenase activity in the presence of up to 50, preferably up to 80 percent by volume of isopropanol, or in the presence of up to 20, preferably up to 50 percent by volume of acetone.
16. The use of a biocatalyst with alcohol dehydrogenase activity according to any one of claims 1 to 15 in the oxidation of secondary alcohols and/or the reduction of ketones.
17. The use according to claim 16, where the oxidation and/or reduction is one of the reactions in the following reaction scheme (A)



wherein, in formula I, Ia and Ib,  $R_1$  and  $R_2$  are two different moieties from the group consisting of unsubstituted or substituted alkyl, unsubstituted or substituted alkenyl, unsubstituted or substituted alkynyl, unsubstituted or substituted cycloalkyl, unsubstituted or substituted aryl and unsubstituted or substituted heterocyclyl, or  $R_1$  and  $R_2$  together form an unsubstituted or substituted bridge; and in formula III and formula IV,  $R_3$  and  $R_4$  are two different or preferably two identical lower alkyl or aryl moieties, or together form a bridge.

18. The use according to claim 16, where the oxidation is one of the reactions in the following reaction scheme (B):

reaction scheme B (oxidation of diols):

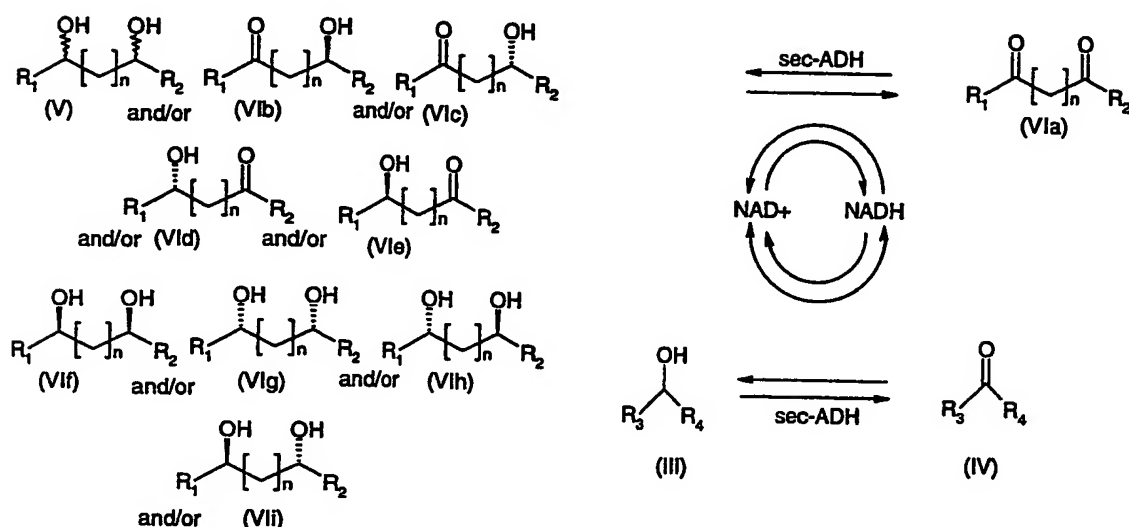


wherein

$n$  is a whole number in the range from 0 to 20, and  $R_1$ ,  $R_2$ ,  $R_3$  and  $R_4$  have the same meaning as described in claim 17.

19. The use according to claim 16, where the reduction is one of the reactions in the following reaction scheme (C):

reaction scheme C (reduction of diketones):



wherein

$n$  is a whole number in the range from 0 to 20, and  $R_1$ ,  $R_2$ ,  $R_3$  and  $R_4$  have the same meaning as described in claim 17.

20. The use according to anyone of claims 16 to 19 in the stereoselective production of chiral or diastereomeric alcohols from the corresponding ketones.
21. The use according to anyone of claims 16 to 19 in the chemoselective, especially stereoselective, oxidation of secondary alcohols where only the hydroxy group with the appropriate steric form is oxidized to the corresponding oxo group
22. The use according to claim 21 where the stereoselective oxidation is used for the enantioselective oxidation of only one isomer of a mixture of enantiomers or diastereomers of secondary alcohols.



23. A nucleic acid coding for a biocatalyst with alcohol dehydrogenase activity according to any one of claims 1 to 15.
24. A nucleic acid according to claim 23, especially a DNA or RNA, which is a recombinant nucleic acid.
25. A microorganism transformed with a nucleic acid coding for a biocatalyst according to any one of claims 1 to 15
26. The use of a microorganism according to claim 25 in the production of a biocatalyst according to any one of claims 1 to 15.
27. The use of a microorganism, especially a host cell expressing the biocatalyst, according to claim 25 in the catalysis of the oxidation of secondary alcohol or the reduction of ketones, especially according to any one of claims 16 to 19.

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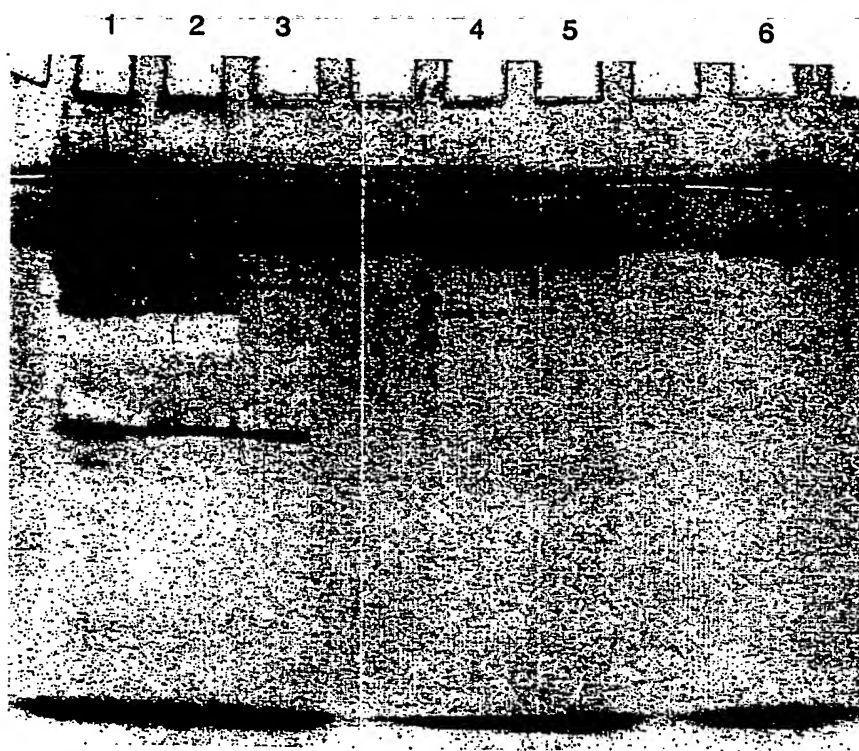


Fig. 1

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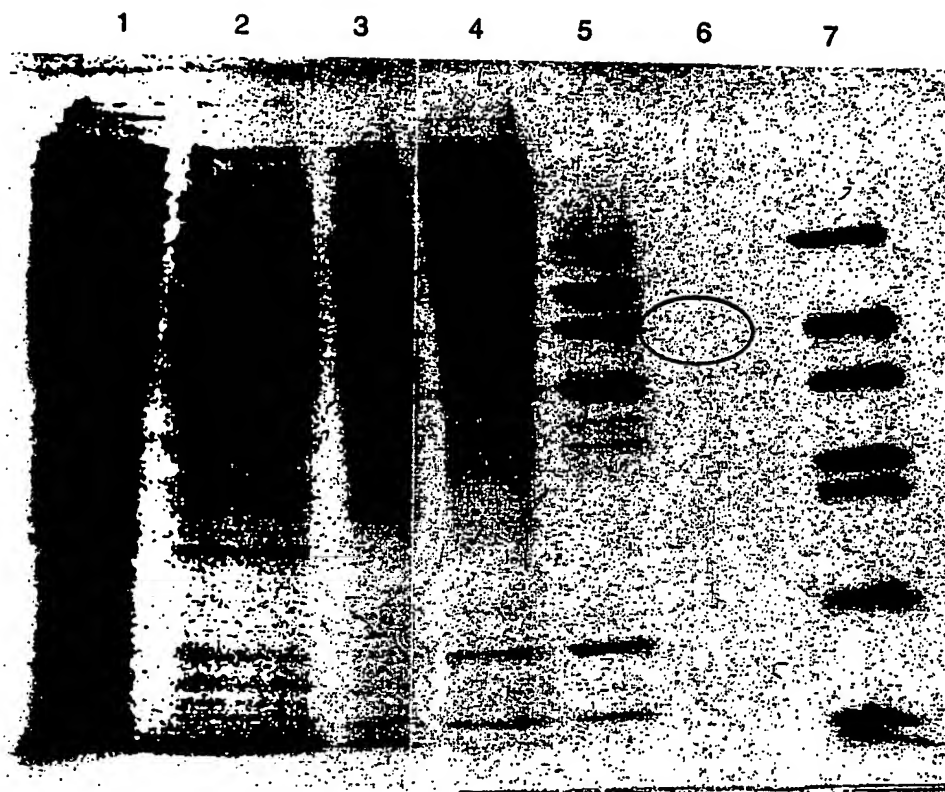


Fig. 2

- 1 -

## SEQUENCE LISTING

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- 3 -

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&lt;223&gt; Xaa in position 3 is either isoleucine or leucine

&lt;220&gt;

&lt;221&gt; UNSURE

&lt;222&gt; (9)

&lt;223&gt; Xaa in position 9 is either isoleucine or leucine

&lt;400&gt; 6

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1 5 10

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 03/02439

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/04 C12N15/53

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, WPI Data, PAJ, SEQUENCE SEARCH, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>STAMPFER W. ET AL.: "Biocatalytic Asymmetric Hydrogen Transfer" ANGEW. CHEM. INT. ED., vol. 41, no. 6, 15 March 2002 (2002-03-15), pages 1014-1017, XP001093791 the whole document and in particular page 1014, right-hand column, last paragraph</p> <p style="text-align: center;">--- -/-</p>	1-27

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

23 July 2003

Date of mailing of the international search report

31/07/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Ury, A

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 03/02439

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HIRATA T ET AL: "A 38 kDa allylic alcohol dehydrogenase from the cultured cells of <i>Nicotiana tabacum</i> " PHYTOCHEMISTRY, PERGAMON PRESS, GB, vol. 55, no. 4, October 2000 (2000-10), pages 297-303, XP004291646 ISSN: 0031-9422 abstract; figure 1 page 297, right-hand column, last paragraph	1-6, 14-27
X	KOSTICHKA KRISTY ET AL: "Cloning and characterization of a gene cluster for cyclododecanone oxidation in <i>Rhodococcus ruber</i> SC1." JOURNAL OF BACTERIOLOGY, vol. 183, no. 21, November 2001 (2001-11), pages 6478-6486, XP002214988 ISSN: 0021-9193 * Whole document, and in particular page 6478, right-hand column, lines 19-27; page 6483, left-hand column, lines 29-32; Table 1 *	1-4,7, 14-27
X	US 6 255 092 B1 (MATSUYAMA AKINOBU ET AL) 3 July 2001 (2001-07-03) column 2, line 22-61; claim 1	1-5, 14-27
X	KIM TAE-KANG ET AL: "Purification and characterization of a cyclohexanol dehydrogenase from <i>Rhodococcus</i> sp. TK6." JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY, vol. 12, no. 1, February 2002 (2002-02), pages 39-45, XP001106020 ISSN: 1017-7825 abstract	1-6, 14-22
P,A	POGOREVC M ET AL: "Enantioselective stereoinversion of sec-alkyl sulfates by an alkylsulfatase from <i>Rhodococcus ruber</i> DSM 44541" TETRAHEDRON: ASYMMETRY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 13, no. 13, 19 July 2002 (2002-07-19), pages 1435-1441, XP004374319 ISSN: 0957-4166 page 1436, right-hand column, line 12 - line 16	1-27

-/-



# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 03/02439

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SCHENKELS P ET AL: "Nicotinoprotein (NADH-containing) alcohol dehydrogenase from Rhodococcus erythropolis DSM 1069: an efficient catalyst for coenzyme-independent oxidation of a broad spectrum of alcohols and the interconversion of alcohols and aldehydes."</p> <p>MICROBIOLOGY (READING, ENGLAND) ENGLAND APR 2000, vol. 146 ( Pt 4), Apr11 2000 (2000-04), pages 775-785, XP002214989 ISSN: 1350-0872 * page 775; "Introduction" *</p>	1-27
A	<p>REID M F: "Molecular Characterization of Microbial Alcohol Dehydrogenases"</p> <p>CRITICAL REVIEWS IN MICROBIOLOGY, CRC PRESS, INC., BOCA RATON, FL, US, vol. 20, no. 1, 1994, pages 13-56, XP002110760 the whole document</p>	1-27

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 03/02439

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6255092	B1	03-07-2001	
		JP 7231785 A	05-09-1995
		EP 0645453 A2	29-03-1995
		US 5763236 A	09-06-1998